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Review

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Histone deacetylase inhibitors merged with protein tyrosine kinase inhibitors

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Summary **Histone deacetylases (HDACs) are a family of metal enzymes which mainly regulates the acetylation level of histone, together with histone acetyl transferases (HATs). Recently, because many HDAC inhibitors (HDACis) have entered clinical trials for both solid and liquid tumors, HDACs are recognized as one of the promising targets for cancer treatment. The current trend is that more and more HDAC inhibitors are used in combination with other antitumor agents in order to optimize their effect and toxicity. Protein tyrosine kinases (PTKs) which play important roles in cellular signal transduction pathways and regulate series of physiological and biochemical processes, are another family of hot antitumor targets. This brief review will mainly talk about several reported chimeric HDACs-PTKs inhibitors.**

Keywords: HDAC, PTKs, chimeric inhibitor

1. Introduction

Histone deacetylases (HDACs) are a class of proteases widely existing in eukaryotic cells. Up to now, there are 18 human HDACs that have been identified. On the basis of sequence similarity, intracellular localization and tissue specific distribution, human HDACs have been classified into 4 groups (*1*). Class I HDACs (HDAC1, 2, 3, and 8) located in the nucleus are expressed widely in various tissues and inhibit gene expression. Based on the sequence homology and domain organization, Class II HDACs are divided into two sub-groups, Class IIa (HDAC4, 5, 7, and 9) and Class IIb (HDAC6 and 10), and may be associated with cell differentiation (*2,3*). Class IIa HDACs shuttle between the cytoplasm and nucleus; Class IIb HDACs situated in cytoplasm typically contain double zinc catalytic centers. Class IV HDAC (HDAC11) is comprised of a unique member HDAC11 just because of the specific structure of HDAC11 (*4*). In contrast to all of the above Zn^{2+} dependent HDACs, Class III (Sir1-7) homologous to the yeast Sir2 family of proteins are also called sirtuins (*2,3,5*).

The nucleosome, which is the basic structural unit of eukaryotic chromosomes, consists of an octamer (an H3-H4 tetramer and two H2A-H2B dimers) and DNA (deoxyribonucleic acid) wrapping around the octamer (*6*). It is the HDACs that catalyze the cleavage of the N-acetyl group from acetylated lysine residues located on the tails of the core nucleosomal histones (*7*). Together with the histone acetyl transferases, HDACs regulate the balance of acetylation and deacetylation (*8*). When HDACs are more active, histone proteins are deacetylated and bind tighter to DNA, thus it is difficult for transcriptional regulatory proteins to combine with DNA, leading to the inhibition of gene transcription (*9*). In addition to the regulation of histones, HDACs can also regulate the acetylation status of a variety of non-histone substrates, including key tumor suppressor proteins and proteins expressed by oncogenes (*10*).

Since vorinostat (SAHA) was approved by the US Food and Drug Administration (FDA) for cutaneous T-cell lymphoma in 2006, pharmaceutical chemists have paid increasing attention to HDACis (histone deacetylase inhibitors) in cancer therapy. According to the previous study, we easily find that almost all HDACis have the common pharmacophore, containing three domains. One is ZBG short for the zinc-binding group, such as hydroxamic acid. Another is a cap group, which is generally a hydrophobic and aromatic group. It is a saturated or unsaturated linker domain, composed of linear or cyclic structures that connects the ZBG and the cap group. According to their chemical structures, HDACis can be mainly divided into several classes,

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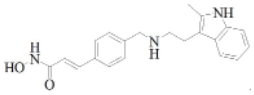
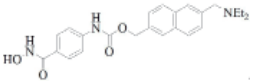
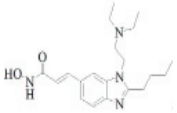
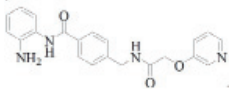
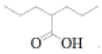
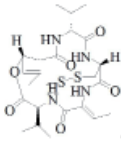
Table 1. Some typical clinical HDAC inhibitors

Class	HDAC inhibitors	Structure	Clinical trials	*Ref.*
Hydroxamic acid	Vorinostat (SAHA)		Approved	(*11*)
	Panobinostat (LBH-589)		Approved	(*12-15*)
	ITF2357		Phase II	(*16*)
	SB939		Phase II	(*17*)
Benzamide	Entinostat (MS-275)		Phase II	(*8*)
Carboxylic acid	Valproic acid		Phase III	(*18*)
Cyclic peptide	Romidepsin (FK228)		Approved	(*19*)

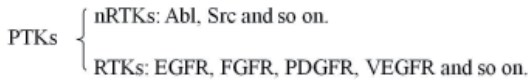


Figure 1. The family of PTKs and some typical members.

including hydroxamic acid, benzamide, carboxylic acid and cyclic peptides (Table 1).

Protein tyrosine kinases can catalyze the transfer of the γ-phosphoryl group on the ATP molecule to the tyrosine residues of substrate proteins, making them phosphorylated (*16*). The protein tyrosine kinase family with over 90 members is divided into two subfamilies, the receptor tyrosine kinases (RTKs) and the non-receptor tyrosine kinases (nRTKs) (Figure 1) (*21,22*). RTKs include EGFR (epidermal growth factor receptor), FGFR (fibroblast growth factor receptor), VEGFR (vascular endothelial growth factor receptor) and so on. nRTKs include Abl, Src and so on. FDA has already approved imatinib, gefitinib, erlotinib, sorafenib, sunitinib, dasatinib, nilotinib, laptinib, and pazopanib for clinical use (Table 2) (*23*). All of the RTKs are membrane proteins which consist of three parts: an extracellular ligand-binding domain, a transmembrane region and an intracellular kinase domain (*22,24*). After a ligand binds to the extracellular domain, the receptors form dimers stimulating catalytic activity, then several tyrosine residues of the intracellular kinase domain become autophosphorylated. Only autophosphorylated RTKs can activate downstream signaling pathways.

Her (human epidermal growth factor receptor) has four subfamily-EGFR/Her1, Her2-4. EGFR is one typical target for kinase inhibitors in clinical trials. EGFR is a trans-membrane protein belonging to the HER-family of RTKs and can be activated by binding to EGF (epidermal growth factor), TGF-alpha (alpha-transforming growth factor) and amphiregulin (*34*). The overexpression of both receptors and ligands may lead to uncontrolled activation of signal transduction pathways. However, Her2 has no soluble ligand, which is called an orphan receptor, and it is easier to form heterogeneous dimers with the other three family members. As Table 2 shows, gefitinib, erlotinib, and laptinib mainly target EGFR and Her2.

Numerous combinations of HDACis with other cytotoxic or targeted therapeutics have been tested in clinical trials (Table 3). There is no doubt that a synergistic effect really exists for inhibiting these two kinds of targets. For instance, a combination of SAHA and erlotinib cause a synergistic inhibition of cell growth. Tumor cells are potentially vulnerable to HDAC6 inhibition and this has been exploited by combining HDACi with proteosome inhibitors such as bortezomib. In addition, the combined effect of directly targeting oncogenic client proteins using imatinib and destabilizing Bcr-abl by HDACi has a potent antitumor effect *in vitro*. Moreover, the chemical flexibility of HDACis makes it possible to design multi-targeted small molecules. First of all, HDACis and EGFRis can be simply connected via a linker that can be cleaved under

Table 2. Kinase inhibitors approved for use

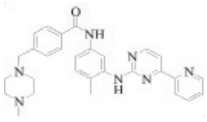
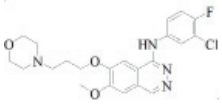
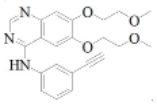
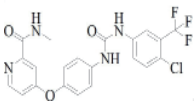
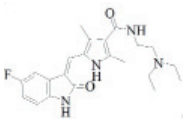
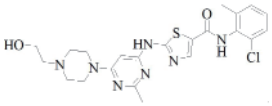
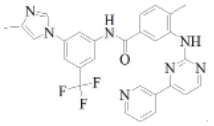
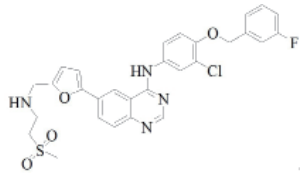
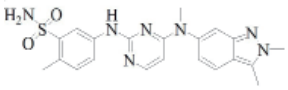
Generic name	Structure	U.S. FDA approved indications	Target kinases	*Ref.*
Imatinib (2001)		CML	Abl, c-Kit, PDGFRa, PDGFRb	(*25*)
Gefitinib (2003)		NSCLC	EGFR	(*26*)
Erlotinib (2004)		NSCLC, pancreatic cancers	EGFR	(*27*)
Sorafenib (2005)		Hepatocellular carcinoma, renal cell carcinoma	Raf, VEGFR2, VEGFR3, c-Kit, PDGFRb	(*28*)
Sunitinib (2006)		GIST, renal cell carcinoma	c-Kit, VEGFR, PDGFR, FLT3	(*29*)
Dasatinib (2006)		CML	Abl, c-Kit, PDGFR, Src	(*30*)
Nilotinib (2007)		CML	Abl, c-Kit, PDGFRb, Src, Ephthrin	(*31*)
Laptinib (2007)		Breast cancer	EGFR, Her-2	(*32*)
Pazopanib (2009)		Kidney cancer	VEGFR	(*33*)

Table 3. Active clinical trials using HDACi in combination with Protein Tyrosine Kinase Inhibitors

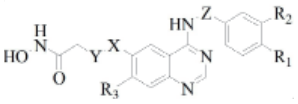
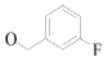
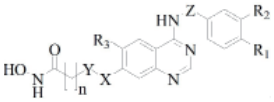
HDACi	Phase	Combination drug	Indication
SAHA	II	Bortezomib	Recurrent glioblastoma multiforme
SAHA	II	Bortezomib	Recurrent lymphoma
SAHA	II	Bortezomib	Refractory multiple myeloma
SAHA	III	Bortezomib	Multiple myeloma
LBH589	I	Bortezomib	Multiple myeloma
Depsipeptide	I/II	Bortezomib	Multiple myeloma
Depsipeptide	II	Bortezomib	Multiple myeloma
PXD101	I	Bortezomib	Advanced solid tumors, lymphomas

physiological conditions. In addition, without interfering each other, two individual moieties can be joined through a stable spacer allowing interaction with their proposed targets. Third, two pharmacophores can lead to a single compound whose fragments attack the desired targets without interference. Thus, it is rational to target both histone deacetylase and protein tyrosine kinase.

2. Examples of multi-targeting HDACis

CUDC-101 is one successful example in clinical phase I (*35,36*). Cai *et al*. used a suitable linker to connect the known EGFR/Her2 inhibitor quinazoline with the known HDAC inhibitor hydroxamic acid. They synthesized a series of compounds with CUDC-101 as

Table 4. The compounds Cai *et al*. designed

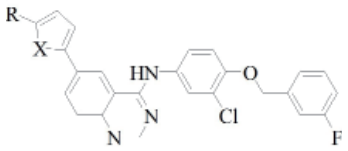
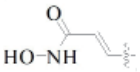
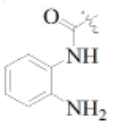
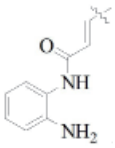
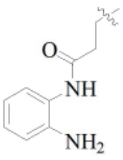
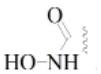
Compound	X	Y	Z	n	R_1	R_2	R_3
							
1	O	CH_2	direct	1	F	Cl	OCH_3
2	O	CH_2	direct	2	F	Cl	OCH_3
3	O	CH_2	direct	3	F	Cl	OCH_3
4	O	CH_2	direct	3	H	C≡CH	OCH_3
5	O	CH_2	direct	4	F	Cl	OCH_3
6	O	CH_2	direct	4	H	C≡CH	OCH_3
7	O	CH_2	direct	5	F	Cl	OCH_3
8 CUDC-101	O	CH_2	direct	5	H	C≡CH	OCH_3
9	O	CH_2	direct	5	F	C≡CH	OCH_3
10	O	CH_2	direct	5		Cl	OCH_3
11	O	CH_2	direct	5	F	Cl	H
12	O	CH_2	direct	5	F	Cl	$CH_2CH_2OCH_3$
13	NH	C=O	direct	6	F	Cl	OCH_3
14	S	CH_2	direct	5	F	Cl	OCH_3
15	$S(O)_2$	CH_2	direct	5	F	Cl	OCH_3
16	O	CH_2	CH_2	5	F	Cl	OCH_3
17	O	CH_2	CH_2	5	H	H	OCH_3
18	O	CH_2	CH_2	5	F	H	OCH_3
19	O	CH_2	(S)- CHCH3	5	H	H	OCH_3
20	O	CH_2	(R)- CHCH3	5	H	H	OCH_3
21	O	CH_2	(R)- CHCH3	5	F	H	OCH_3
22	O	CH_2	(R)- CHCH3	5	Cl	H	OCH_3
							
23	O	CH_2	direct	5	F	Cl	OCH_3
24	O	CH_2	direct	5	H	C≡CH	OCH_3

a potent candidate (Table 4).

To investigate structure activity relationships, compounds were evaluated in EGFR/Her2 kinase and HDAC enzyme assays. The data showed with an increased carbon chain length, HDAC inhibitory activity increased and the optimal length was six. Apparently, the length of the hydroxamic acid side chain was important for HDAC inhibition. Phenyl ring substitutions almost had no effect on HDAC inhibition activity, but larger ones such as compound **10** showed decreased activity. However, the carbon chain length and phenyl ring substitutions were not critical factors that affected EGFR/Her2 activity. Taking the influence of C-7 substitutions into consideration, they chose six carbons as the linker and synthesized compounds **7**, **11**, **12**. When X is O, it was amazing that on one hand, there was no apparent differences for inhibiting HDAC, on the other hand, as for EGFR/Her2 inhibition, OCH_3 was superior to $CH_2CH_2OCH_3$, which was more efficient than H. Then fixing OCH_3 as R_3, **7**, **13**, **14**, **15** were compared. **7** (X=O) showed outstanding HDAC inhibition. However, it was a pity that rarely could we tell **7**, **13**, **14** apart only according to EGFR/Her2 inhibition, indicating that either, sulfide and amide may not be critical. The conjugation of the quinazoline ring with the phenyl ring was disrupted when the length between them was longer. Only compound **17** (Z= CH_2, R_1 = H, R_2 = H) and **18** (Z = CH_2, R_1 = F, R_2 = H) were promising on HDAC inhibition. With regard to EGFR/Her2 inhibition, all compounds were inferior to **7**. In brief, the changing of an aniline to a benzylamine resulted in less potent compounds. Supposing that the hydroxamic acid side chain position may have an effect on inhibitory activities, **23** and **24** were synthesized and behaved less potently. In conclusion, **7** and **8** were better HDAC, EGFR and Her2 inhibitors. From these conditions, they further evaluated these two compounds against several cancer cell lines, such as NSCLC (nonsmall-cell lung cancer), liver, breast and pancreatic cancer cell lines. Compared with positive control compounds-vorinostat, erlotinib, lapatinib, the combination of vorinostat and erlotinib, and the combination of vorinostat and lapatinib, it is stirring that **8** behaved much better or at least equally (*35*).

Cheng-Jung Lai *et al*. continued to study the antiproliferative effects of **8**. They tested the inhibition of HDAC, EGFR and Her2 enzyme activities, indicating nearly the same result. Additionally, by exposing cells to different concentrations of CUDC-101 for 5-24 h, they evaluated the effects in cancer cell lines. Fortunately,

Table 5. The compounds depending on laptinib which Siavosh Mahboobi *et al*. designed

Compound	General formula	R	X
25a			O
25b			S
25c			CH=CH, R in position meta
25d			CH=CH, R in position para
26a			O
26b			S
26c			O
26d			CH=CH, R in position meta
26e			CH=CH, R in position para
26f			CH=CH, R in position para
27a			O
27b			S

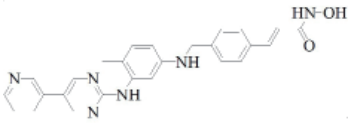
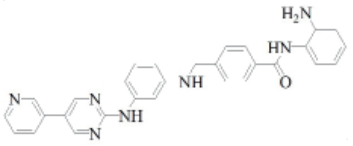
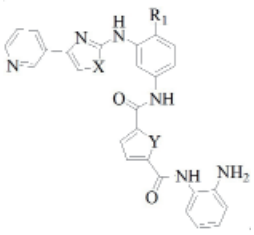
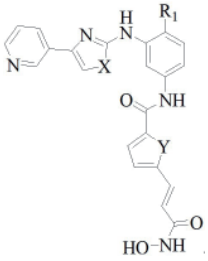
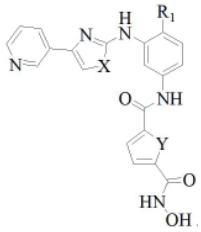
CUDC-101 could increase the acetylation of not only histone H3 and H4 but also non-histone substrates such as p53 and α-tubulin, and inhibit the autophosphorylation of EGFR and Her2. This result made it clear that CUDC-101 really targeted HDAC, EGFR, and Her2. The data of growth inhibition *in vitro* matched what Cai *et al*. observed. It was encouraging that CUDC-101 displayed a broad activity *in vivo* xenograft models. In mouse xenograft models, CUDC-101 induced 30% tumor regression and did better than positive control compound erlotinib at a maximum tolerated dose and vorinostat at an equimolar concentration (*36*).

In short, in view of its potent drug-like properties and safety, CUDC-101 has already been selected for clinical development. Siavosh Mahboobi *et al*. designed and synthesized another series of histone deacetylase inhibitors merged with protein tyrosine kinase inhibitors depending on the compound laptinib. SAHA a hydroxamic acid-based HDAC inhibitor has already been approved and MS-275 as a benzamide-HDAC inhibitor has been in phase II clinical trial (Table 1), so hydroxamic acid and benzamide were chosen to chelate the Zn^{2+}. They also changed the substitution patterns from meta to para, altered bioisosteres and explored the effect on the system of vinylogous (Table 5).

In biochemical, cellular and target-specific assays, they concluded several points. First, hydroxamic acid displayed more potent HDAC inhibition, meanwhile, hydroxamic acid or benzamide were insignificant for EGFR/Her2 inhibition. Second, for HDAC inhibition, bioisosteres and vinylogous were of prime importance and meta surpassed pera. The fact that almost all of the compounds behaved no better than SAHA really depressed everyone. On the contrary, even though bioisosteres, vinylogous and substitution patterns had nothing to do with EGFR/Her2 inhibition, compared to laptinib, each compound showed expectant ability for inhibiting EGFR/Her2. In addition, they also investigated cytotoxicity of these compounds towards some selected cancer cell lines and found that **25a** and **25c** were the most potential chimeric inhibitors (*37*). From these preclinical data, some improvements could be seen noticeably, but HDAC inhibition activity was far from satisfactory. More effort should be made for further development.

Based on the compound imatinib, Siavosh Mahboobi *et al*. designed and synthesized another series of histone deacetylase inhibitors (Table 6). *In vitro* data, benzamide almost showed no HDAC6 inhibition and benzamide was outstripped by hydroxamic acid in inhibiting HDAC1. All the designed compounds were exciting because of their potent inhibition of HDAC. On the contrary, their inhibition of Abl kinase in comparison with imatinib was just passable. The introduction of cellular histone H3 K(Ac) hyperacetylation and cytotoxicity towards HeLa (henrietta lacks strain of

Table 6. The compounds depending on imatinib which Siavosh Mahboobi *et al*. designed

Compound	General formula	R_1	X	Y
28				
29				
30a		H	CH=N	CH=CH
30b		CH_3	CH=N	CH=CH
31b		CH_3	CH=N	CH=N
32b		CH_3	CH=N	S
33a		H	S	CH=CH
33b		CH_3	S	CH=CH
34a		H	S	CH=N
34b		CH_3	S	CH=N
35a		H	S	S
35b		CH_3	S	S
36a		H	S	CH=CH
36b		CH_3	S	CH=CH
37a		H	S	S
37b		CH_3	S	S
38a		H	S	CH=CH
38b		CH_3	S	CH=CH

cancer cells) and K562 cells were used to estimate the cellular efficacy. All HDAC1 inhibitors had a potency of inducing histone H3 hyperacetylation in HeLa cells. In addition, the cytotoxicity of these analogues towards HeLa and K562 cells was in low micromolar or sub-micromolar range, nevertheless, the potency of imatinib towards K562 cells was superior to that of all designed compounds. Replacing the amine structure with amide resulted in reduced activity. In addition, introduction of a heterocycle decreased Abl kinase inhibition. From the benzene derivatives to pyridine and thiophene derivatives the HDAC1 inhibitory potency decreased. Although **30b**, **32b**, and **36b** showed potent HDAC inhibition, their inhibition of Abl kinase was low grade compared with imatinib (*38*).

Miao Zuo *et al*. synthesized N-aryl salicylamides as novel HDAC-EGFR dual inhibitors (Table 7). As is known, the length of the hydroxamic acid chain is a key factor for EGFR and HDAC inhibition and the optimal length is six or five. From the data of inhibition activities against EGFR, **39b**, **39h**, **39i**, **39l**, **39k**, and **39o** had strenuous inhibition activities, and the following 4 points were easily found. Comparing **39f**, **39m**, and **39n**, **39f** behaved much better just because of the construction of a pseudo six-membered ring. And **39n** played no important role. Moreover, it was favorable when R_2 was a methoxy group. Comparing **39o** with **39f**, there was no doubt that an ether linker was much better than an amide linker, which was also applied to HDAC inhibition. As for HDAC inhibition, it was found that only compound **39n** was superior to SAHA. Thus the substitution on the N-phenyl was not vital. The antiproliferative activities were evaluated against human cancer cell lines A431, A549, and HL-60. **39o** did best against A431 and A549. At the same time, **39k** and **39n** exhibited higher potency against HL-60. All in all, N-aryl saliylamides with a hydroxamic acid moiety at the 5-position were promising as HDAC-EGFR dual inhibitors (*39*).

Based on erlotinib, Thomas Beckers *et al*. designed a new series of dual-selective inhibitors (Table 8). They

Table 7. The compounds which Miao Zuo *et al*. synthesized

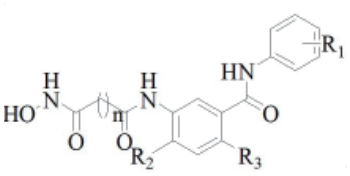
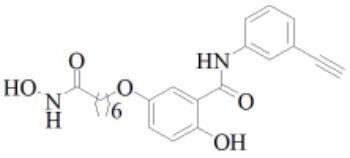
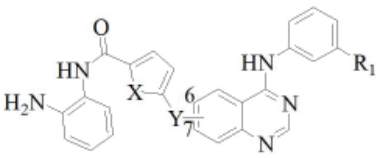
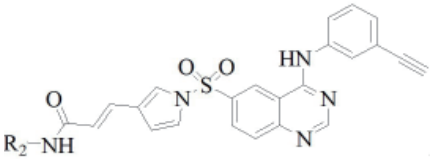
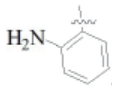
Compound	General formula	n	R_1	R_2	R_3
39a		5	H	H	OH
39b		5	3-F	H	OH
39c		5	3,4-diF	H	OH
39d		5	3-Cl-4-F	H	OH
39e		5	3-CF_3	H	OH
39f		5	3-C≡CH	H	OH
39g		5	3,4-diCl	H	OH
39h		5	3-CF3-4-Cl	H	OH
39i		5	3-Cl-4-(3-FBnO)	H	OH
39j		6	3-C≡CH	H	OH
39k		5	3-Cl-4-F	OCH_3	OH
39l		5	3-C≡CH	OCH_3	OH
39m		5	3-C≡CH	H	H
39n		5	3-C≡CH	H	OCH_3
39o					

Table 8. The compounds which Miao Zuo *et al*. synthesized

Compound	General formula	X	Y	R_1/R_2
40a		CH=CH	6-NHCO	C≡CH
40b		N=CH	6-NHCO	C≡CH
40c		S	6-NHCO	C≡CH
41a		CH=CH	7-NHCO	C≡CH
41b		N=CH	7-NHCO	C≡CH
41c		S	7-NHCO	C≡CH
42a		CH^2	6-OCH_2	Br
42b		CH^2	6-OCH_2	C≡CH
43				
44				OH

tested the inhibition of nuclear extract, cell HDAC and rHDAC1, rHDAC3, rHDAC6 and rHDAC8. 40**a-c** had no significant inhibition of HDAC. However, **41a-c** with substitution at the 7-position achieved a better performance. Compared to SAHA, **41a-c** were far from satisfactory. **42a** and **42b** almost had the same inhibition, showing that exchange of the ethyl group with bromine made no difference. As for **43** and **44**, they behaved better and **44** was a great surprise to us with a lower nanomolar inhibition.

In view of HDAC inhibition, only **42a**, **42b**, **43**, and **44** were tested for inhibition of EGFR and Her-2 kinase activity. To some extent, all of them inhibited EGFR and HER2, and **42a**, **42b**, and erlotinib were almost at the same level. Unfortunately, the selectivity profile towards EGFR/Her1 and Her2 did not obviously change.

With regard to cytotoxicity, **42a**, **42b**, **43**, and **44** showed profound activity towards A549 NSCLS cells with **44** being the most promising dual inhibitor (*40*).

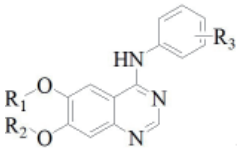
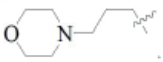
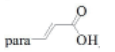
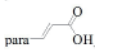
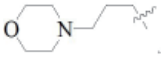
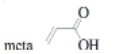
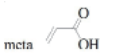
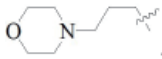
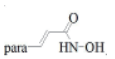
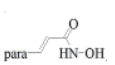
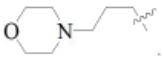
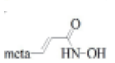
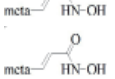
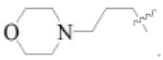
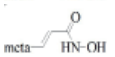
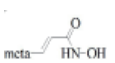
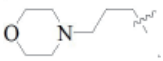
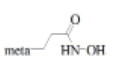
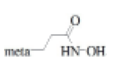
Xuan Zhang *et al*. designed other dual-targeted inhibitors (Table 9). Owing to the hydroxamic acid being a potent zinc binding group, **45a**, **45b**, **46a**, and **46b** with carboxylic acids showed no HDAC inhibition. The ZBG (zinc binding group) location played an important role in unsaturated hydroxamates, while it had no influence on the saturated ones. **48a** was the best HDAC inhibitor.

Unfortunately, all the compounds were inferior to lapatinib on EGFR inhibition and Her2 inhibition, suggesting that hydroxamate on the phenyl group negatively affected PTK inhibition. Thus lipophilic ZBG might be worthy of investigation (*41*).

3. Conclusions

Recently, HDACis have been recognized as a promising

Table 9. The compounds which Xuan Zhang *et al*. synthesized

Compound	General formula	R_1	R_2	R_3
45a			CH_3	
45b		$CH_3OCH_2CH_2$	$CH_3OCH_2CH_2$	
46a			CH_3	
46b		$CH_3OCH_2CH_2$	$CH_3OCH_2CH_2$	
47a			CH_3	
47b		$CH_3OCH_2CH_2$	$CH_3OCH_2CH_2$	
48a			CH_3	
48b		$CH_3OCH_2CH_2$	$CH_3OCH_2CH_2$	
49a			CH_3	
49b		$CH_3OCH_2CH_2$	$CH_3OCH_2CH_2$	
50a			CH_3	
50b		$CH_3OCH_2CH_2$	$CH_3OCH_2CH_2$	

class of anticancer therapeutics and many chimeric HDACis have been synthesized, including inhibitors merged with protein tyrosine kinase inhibitors. Their chemical flexibility made it possible that HDACis can bind to multi-targets. However, when contrasted to the comparable compound which aims at one of the targets, the chimeric HDACis may have a disadvantage that they are less potent. Besides, more clinical studies are needed to decide whether they can combat drug resistance. In a word, such studies encourage people to do more on HDAC inhibitors merged with protein tyrosine kinase inhibitors.

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Review

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Multidrug resistant tuberculosis treatment in India

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Summary **Multidrug-resistant tuberculosis (MDR-TB) is posing a major public health threat as well as a big challenge to global efforts of tuberculosis control. The management of MDR-TB patients is difficult, expensive, challenging and quite often leads to treatment failure. To avoid further transmission, a comprehensive approach for rapid detection, proper treatment and effective public health measures must be ensured. It must also be emphasized that even optimal treatment of MDR-TB will not alone curb the epidemic. Efforts must be focused on the effective use of first-line drugs in every new case so as to prevent the ultimate emergence of MDR-TB.**

Keywords: Multidrug-resistant tuberculosis, second line drugs, treatment regimens, adjuvant therapies, treatment outcomes

1. Introduction

Multi-drug Resistant Tuberculosis (MDR-TB) is defined as disease due to *M. tuberculosis* that is resistant to isoniazid and rifampicin with or without resistance to other drugs (the culture and drug susceptibility test result being from an accredited laboratory). It has been an area of growing concern, and is posing a threat to global efforts of tuberculosis control (*1*). Treatment of MDR-TB is difficult, very expensive, challenging and quite often leads to treatment failure. This write up aims to review the treatment of MDR-TB in India.

2. Second line drugs and their dosage

The second line drugs (SLD's) with their dosages used for treatment of MDR-TB are shown below (Table 1). It is generally thought that, SLD's are frequently associated with very high rates of unacceptable adverse drug reactions (ADR's), needing frequent interruption and change of regimen, but in clinical practice, it is observed that these drugs are fairly tolerated. A prospective study of 98 MDR-TB patients treated under a modified strategy of Programmatic Management of Drug Resistant Tuberculosis (PMDT) formerly known as DOTS-PLUS showed that nausea and vomiting occurred in 24.5% of patients followed by hearing disturbances (12.3%), dizziness/vertigo (10.2%) and arthralgia (9.2%). Other side effects observed were gastrointestinal intolerance, hypothyroidism and hepatitis. Agents responsible for these adverse effects were Kanamycin (ototoxicity), cycloserine (headache/psychosis), ethionamide (gastrointestinal intolerance/hypothyroidism) and pyrazinamide (arthralgia/hepatitis) (*2*). One study conducted among 39 patients with MDR-TB reported that 41% of patients experienced some side effects but only 21.1% of patients required stoppage or change of drug (*3*). Thus, it is practically possible to treat the MDR-TB patients with these drugs.

3. Cross-resistance

Cross-resistance has been reported between thioamides and thioacetazone (one way resistance –strains resistant to thioacetazone are susceptible to thioamides, the reverse is seldom the case), kanamycin/amikacin with streptomycin (*4*), rifampicin with rifapentine, and rifabutin (> 70% strains) (*5*) and among various derivatives of fluoroquinolones (*6*). Cross-resistance between the fluoroquinolones is almost complete. Limited evidence suggests that the third-generation fluoroquinolones (notably moxifloxacin) do not have complete cross-resistance with the older generations and may have enhanced clinical benefit due to their low Minimum Inhibitory Concentration (MICs),

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Table 1. Doses of anti-tuberculosis drugs used in MDR-TB

Drugs	Average daily dosage	Daily dosage (mg)		Type of anti-mycobacterial activity	*Ref.*
		Minimum	Maximum		
Group 1 First line oral agents					(*19*)
Pyrazinamide (Z)	25 mg/kg	1200	1500	Bactericidal	
Ethambutol (E)	15 mg/kg	800	1200	Bacteriostatic	
Rifabutin (Rfb)	5–10mg/kg	150	600	Bactericidal	
Group 2 Injectable agents					(*19*)
Kanamycin (Km)	15 mg/kg	750	1000	Bactericidal against	
Amikacin (Am)	15 mg/kg	750	1000	actively multiplying	
Streptomycin (S)	15 mg/kg	750	1000	organisms	
Capreomycin (Cm)	15 mg/kg	750	1000		
Group3 Fluoroquinolones					
Moxifloxacin (Mfx)	7.5–10mg/kg	400	400	Weakly bactericidal	
Ofloxacin (Ofx)	15–20 mg/kg	800	1000		
Levofloxacin (Lfx)	7.5–10mg/kg	500	1000		
Group 4 Oral Bacteriostatic second line drugs					(*19*)
Ethionamide (Eto)	15–20 mg/kg	500–750	1000	Bacteriostatic	
Protionamide (Pto)	15–20 mg/kg	500–750	1000		
Para-aminosalicylic acid (PAS)	200–300mg/kg	10 g	12 g		
Cycloserine (Cs)	10–20 mg/kg	500–750	1000		
Terizidone (Trd)	10–20 mg/kg	500–750	1000		
Group 5 Agents with unclear role in treatment of Drug resistant TB					(*19*)
Bedaquiline	400 mg once daily for 2 weeks, 200 mg thrice weekly for 22 weeks after food			Bactericidal	
Delamanid		100 twice daily		Bactericidal	
Clofazimine (Cfz)	4–5 mg/kg	100	300	Bacteriostatic	
Linezolid (Lzd)		600	1200		
Amoxicillin/clavulanate (Amx/Clv)		500/125Twice a day	1000/250 Twice a day	Weakly bactericidal	
Thioacetazone (T)	150 mg/day	-	-		
Imipenem /Cilastatin (Ipm/Cln) High dose	500–1000 mg/every 6 h	500 every 6 hourly	1000 every 6 hourly		
Isoniazid (High dose H)	16–20 mg/kg	600	900		
Clarithromycin (Clr)	10–15 mg/kg	500 twice a day	500 twice a day	Bactericidal (pH dependent)	

enhanced anti-mycobacterial activity, and improved biochemical structure providing metabolic stability and long half-life, theoretically reducing the selection of resistant mutants. While the clinical benefit of newer-generation fluoroquinolones has been validated in one small retrospective study (*7*), more clinical and laboratory research is needed to understand the extent of fluoroquinolones cross-resistance and its clinical relevance. Cross-resistance has also been reported between ethionamide and INH (*8*), amikacin and kanamycin (*4*), amikacin and capreomycin (*4*). Strains resistant to streptomycin/kanamycin/amikacin are still sensitive to capreomycin. It is ineffective to use two drugs of the same group or to use a drug potentially ineffective because of cross-resistance.

4. General principles for designing treatment regimens for multi-drug resistant tuberculosis

Early suspicion, diagnosis and appropriate treatment of MDR-TB is essential to prevent morbidity, mortality and transmission of MDR-TB. Treatment should ideally be initiated in a specialized centre with standard laboratory facilities. Single drug should not be added to a failing regimen. Intermittent therapy is not effective in MDR-TB and should be avoided. A standardized MDR-TB regimen makes it easier to estimate drug needs, manage, distribute drug stocks and in training of personnel in the treatment of MDR-TB patients. Individually designed regimens are based on the patient's history of past drug use, on drug susceptibility testing (DST) of isoniazid, rifampicin, the second-line injectable agents and a fluoroquinolone. Individualized regimens may achieve higher cure rates than standardized MDR-TB regimens in few settings (*9*). Even when standard regimens are used throughout treatment, patients experiencing severe ADR's will need to have their treatment individualized. Past history of drugs taken by the patient and drugs commonly used in the country and prevalence of resistance to first line and second-line drugs should be taken into consideration when designing a standardized regimen.

Table 2. Grouping of anti-tuberculosis drugs

Group	Anti-tuberculosis Drugs	*Ref.*
Group 1 First-line oral agents	Pyrazinamide (Z); Ethambutol (E); Rifabutin (Rfb)	(*19*)
Group 2 Injectable agents	Streptomycin (S); Kanamycin (Km); Amikacin (Amk); Capreomycin (Cm)	(*19*)
Group 3 Fluoroquinolones	Levofloxacin (Lfx); Moxifloxacin (Mfx); Ofloxacin (Ofx)	(*19*)
Group 4 Oral bacteriostatic 2nd line agents	Ethionamide (Eto); Protionamide (Pto); Cycloserine (Cs); Terizidone (Trd); Para-Aminosalicylic acid (PAS); Para-aminosalicylate sodium (PAS-Na)	(*19*)
Group 5 Drugs with unclear efficacy or unclear role in MDR-TB treatment, not recommended by WHO for routine use in MDR-TB patients	Bedaquiline (Bdq); Delamanid (Dlm); Linezolid (Lzd); Clofazimine (Cfz); Amoxicillin/Clavulanate (Amx/Clv); Imipenem/cilastatin (Ipm/Cln); Meropenem (Mpm); high-dose Isoniazid (high-dose H); Thioacetazone (T); Clarithromycin (Clr)	(*19*)

Regimens should consist of at least four drugs with either certain, or almost certain, effectiveness. If the evidence about the effectiveness of a certain drug is unclear, the drug can be part of the regimen but should not depend upon it for success. Often, more than four drugs may be started if the susceptibility pattern is unknown, effectiveness is questionable for an agent (s) or if extensive, bilateral pulmonary disease is present. Design treatment regimens with a consistent approach based on the hierarchy of the five groups of anti-tuberculosis drugs is shown below (Table 2).

Use any first line oral agent (Group-1) to which isolate is sensitive, use one injectable (Group-2), one fluoroquinolone (Group-3) and add as many second line bacteriostatic agents (Group-4) to makeup at least four effective drugs. Group-5 drugs are not recommended for routine use except where an adequate regimen is impossible with Group 1-4. Do not use ciprofloxacin as an anti-tuberculosis drug. When possible, all the drugs should be given once per day, as the high peaks attained in once-a-day dosing may be more efficacious. Para-amino Salicylic acid (PAS) has traditionally been given in split doses during the day to reduce adverse reactions. The drug dosage should be determined by body weight. The injectable agent should be continued for a minimum of 6 months, and for at least 4 months after the patient first becomes and remains smear negative or culture-negative. The patient's cultures, smears, X-rays and clinical status may also aid in deciding whether or not to continue an injectable agent longer than the above recommendation, particularly in the case of patients for whom the susceptibility pattern is unknown, effectiveness of one or more agents is questionable, or extensive or bilateral pulmonary disease is present (*10*). Treatment of ADR's should be immediate and adequate in order to minimize the risk of treatment interruptions and prevent increased morbidity and mortality due to serious ADR's. The minimum duration of treatment is 18 months after culture conversion. Preferably each dose is given as Directly Observed Therapy (DOT) throughout the treatment. At least therapy should be under direct observation preferably for 3-4 months or until sputum conversion. If directly observed treatment is not possible, adherence to treatment must be ensured by intense health education to patient and family members at start of treatment and during each follow-up visit and other adherence measures like checking empty blister packs. No drug should be kept in reserve and the most powerful drugs (bactericidal) should be used initially and in maximum combination so as to ensure that the first battle is won and won permanently. DST of drugs with high reproducibility and reliability (and from a dependable laboratory) should be used to guide therapy. Do not depend on DST in regimen design for Ethambutol, pyrazinamide, and group 4 and 5 drugs. Pyrazinamide can be used for the entire treatment if it is judged to be effective. Many drug resistant tuberculosis (DR-TB) patients have chronically inflamed lungs, which theoretically produces the acidic environment in which pyrazinamide is active. Alternatively, in patients doing well, injectable pyrazinamide can be stopped if the patient can continue with at least three certain, or almost certain, effective drugs. Use adjunctive measures appropriately, including surgery and nutritional and social support. All measures should be taken to persuade and encourage patients not to stop treatment despite all of its discomfort, because it is the last that stands between patient and death (*11-17*).

5. Programmatic management of drug resistant tuberculosis

Programmatic Management of Drug resistant Tuberculosis (PMDT) is an integral component of a tuberculosis control program to manage MDR-TB implemented through program infrastructure. The strategy is designed to manage MDR-TB using second-line anti-tuberculosis drugs within the Directly Observed

Table 3. Suggested regimen for drug resistant/multidrug resistant tuberculosis before or without or unreliable DST reports

Group	Previous use of ATT	Drug regimen	Duration in months	Drugs given (Responders)	Duration in months	Drugs given (Non- Responders)	*Ref.*
I	Misused drugs like SHE and T	Rifampicin, Isoniazid, Ethambutol Pyrazinamide + Streptomycin	2–3*	Rifampicin Isoniazid Ethambutol + Pyrazinamide	9	Treat as Group II or Take help of DST result from accredited laboratory	(*3*)
II	Misused drugs like SHREZ & T	Streptomycin, Isoniazid, Rifampicin, Ethambutol Pyrazinamide	2–3*	Rifampicin Isoniazid Ethambutol + Pyrazinamide	9	Treat as Group III or Take help of DST result accredited laboratory	(*3*)
III	Failed after adequate 5 drugs (SHREZ)	Kanamycin, Ethionamide, Cycloserine, Pyrazinamide, Ethambutol	6-9	Ethionamide; Fluoroquinolone (Levofloxacin/ Ofloxacin) Cycloserine, Ethambutol	18	Treat as Group IV or Take help of DST result accredited laboratory and consider surgery	(*3*)
IV	Failed on group III treatment	Capreomycin, PAS, Moxifloxacin, High dose-INH, Clofazimine, Linezolid, Amoxycillin/ Clavulanate	6–12	PAS, Moxifloxacin, High dose-INH, Clofazimine, Linezolid, Amoxycillin/ Clavulanate	18	Consider surgery	(*3*)

* depending on sputum conversion can be used for 3-6 months if toxicity does not intervene. R-rifampicin, H-isoniazid, E-ethambutol, Z-pyrazinamide, S-streptomycin, T-thiacetazone.

Treatment and Short course chemotherapy (DOTS) strategy in low and middle-income countries like India. Therefore in every DOTS implementing unit of the country, DOTS would be prioritized above DOTS-PLUS with the view that DOTS reduces the emergence of MDR-TB, and therefore the need for DOTS PLUS over time. MDR-TB patients under programmatic management of drug resistant tuberculosis will be using a standardized treatment regimen known as Category IV regimen, comprised of 6 drugs (kanamycin, levofloxicin, ethionamide, cycloserine, pyrazinamide, and ethambutol) during 6-9 months of the intensive phase and 4 drugs (levofloxacin, ethionamide, cycloserine, and ethambutol) during the 18 months of the continuation phase. PAS is included in the regimen as a substitute drug if any bactericidal drug (kanamycin, levofloxacin, pyrazinamide and ethionamide) or any 2 bacteriostatic (ethambutol and cycloserine) drugs are not tolerated. This regimen is highly suitable for high tuberculosis prevalent nations as well as low to middle income countries like India. Injectable agent should be given at least for 6 months and the whole treatment duration is a minimum of 18 months beyond sputum conversion. Fully standardized second line treatment has shown to be feasible and cost- effective in MDR-TB treatment (*16,18*).

In a setup where most of the time either DST results are not available or if available they may not be reliable. Keeping this fact in mind, depending upon past history of anti-tuberculosis treatment the author himself has used four groups of the regimen in treating resistant/multidrug resistant tuberculosis and found it to be effective (*3*). The suggested regimen by author is mentioned below (Table 3).

6. Duration of treatment

The optimal duration of therapy for MDR-TB has not been clearly established and duration remains questionable. World Health Organization (WHO) guidelines recommend continuing therapy for a minimum of 18 months after culture conversion until there is conclusive evidence to support a shorter duration of treatment. Extension of therapy to 24 months may be indicated in chronic cases with extensive pulmonary damage. This is, however, a highly expensive approach that is difficult to implement in the majority of middle- and low-income countries like India that bear the high burden of MDR-TB. The duration of injectable is also controversial. The 2006 and 2008 WHO guidelines advise at least 6 months or at least 4 months after smear or culture conversion. WHO guideline 2011 advises at least 8 months of injectable. In addition duration has to be decided in correlation with other factors also including other drugs in the regimen, bacteriological status and drug toxicity (*11*).

7. Monitoring of treatment

Monitoring of treatment should be done with bacteriological, radiological and clinical methods. Sputum specimens should be obtained for semi-quantitative smear and culture every month from third month onwards during the intensive phase of therapy. Sputum conversion is defined as two sets of consecutive negative smears and cultures, from samples collected at least 30 days apart. Both bacteriological techniques (smear and culture) should be used to monitor patients throughout therapy. After sputum conversion smear examination and culture are done once in three months until the end of therapy (*11*). If such a large number of smears and cultures for follow-up are not possible, then at least five smears and cultures must be done for follow-up (4, 6, 12, 18 and 24 months), X-rays should be done every 6 months whereas clinical monitoring preferably should be done every month (*3*).

8. Adjuvant therapies

In addition to the administration of antimicrobial drug therapy various other treatment modalities may play a significant role in management of patients. These include nutritional support, surgery, collapse therapy, laser therapy, immunomodulation therapy and gene therapy.

9. Nutritional support

Drug-resistant tuberculosis treatment and care should contain integrated nutritional assessment counseling and support for the duration of the illness. Food support may improve treatment adherence in settings were food insecurity is an important access barrier (*19*). Vitamin B6 (pyridoxine) should be given to all MDR-TB patients receiving cycloserine or terizidone, and a high dosage of isoniazid or linezolid to prevent neurological side effects. Vitamin (especially vitamin A) and mineral supplements can be given in areas where a high proportion of the patients have these deficiencies. If multivitamins and minerals (zinc, iron, calcium, *etc*.) are given, they should be dosed three to four hours apart from the fluoroquinolones, as these can interfere with the absorption of these drugs (2*0*).

10. Surgery

The most common operative procedure is resection surgery. It is adjunct to chemotherapy and should not be considered as last resort. Chemotherapy should be given at least for two months prior to surgery and continued for 12-24 months after resection. It is indicated in patients who remain sputum positive, with resistance to a large number of drugs; and localized pulmonary disease (*11*).

11. Collapse therapy

It is a reversible surgical therapy that involves collapse of lung by artificial pneumo-peritoneum or pneumothorax used for cavity containing diseased lung with concept that compression of the cavity will change the local environment in a manner that will inhibit the mycobacteria. This therapy does not appear to be of general utility, although artificial pneumo-peritoneum and pneumothorax may be helpful in highly selected cases (*21*). However, controlled studies are lacking.

12. Laser therapy

This has also been tried as an adjunct to chemotherapy in some countries such as Russia for the treatment of drug resistant TB. This is effective in multi cavitary disease with heavy bacterial loads particularly when there is an increased chance of failure of medical treatment. It is thought to have a role in the rapid killing of bacteria, increases and improves penetration of anti-tubercular drugs in walled off lesions and helps in early closure of cavities and is of proven benefit in tracheal and bronchial stenosis due to endobronchial growth. It also reduces the trauma of surgery and post-operative complications.

13. Immunotherapy or immunomodulation

Therapeutic modulation of the immune system to enhance the host's immunity to control tuberculosis and to shorten the durations of chemotherapy required to 'cure' patients with drug susceptible disease has been tried with some success. *Mycobacterium vaccae* have shown transiently favorable results when given to drug resistant tuberculosis patients that had failed chemotherapy (*22*). Immunomodulation can be affected by enhancing pro inflammatory cytokines like IL-2, IL-12, IFN-γ, TNF- α, inhibiting the anti inflammatory cytokines like IL-4, IL-5, IL-10, addition of serum to enhance humoral factors or diverting the harmful Th2 immune pathway to the beneficial Th1 response by vaccination utilizing *M. vaccae*. However, these therapies are adjuncts and have proved useful in selected cases of drug resistant tuberculosis and randomized control trials, but have failed to confirm the utility of this therapy (*23*). Beneficial effect of parenterally used interferon gamma (IFN- γ) has been reported in disseminated disease attributable to mycobacteria other than tuberculosis that was refractory to chemotherapy (*24*). Favorable results were reported following one-month use of inhaled IFN- γ, 500 micrograms thrice weekly. Interleukin-2 (IL-2) was used to restore antigen responsiveness, presumably *via* enhancing IFN- γ production. Thalidomide has been shown to inhibit the *in vitro* release of TNF- α from peripheral blood monocytes. In patients with active tuberculosis it induces a significant gain in weight (*25*). However the

possibility that thalidomide agents may ameliorate tissue injury in tuberculosis needs further study (*26,27*). The potential role of diverse agents such as transfer factor, indomethacin, and levamisole is yet to be established (*28*). Levamisole as adjunct to drug treatment has been reported to cause more rapid radiological clearing in the treated group. However, it did not significantly affect the clinical outcome (*29*). *Mycobacterium w* (commercially available as Immuvac) has been extensively studied as an effective immunomodulator for treatment of leprosy. It enhances bacterial killing and lesion clearance when used as an adjuvant to multi-drug therapy for leprosy. *Mycobacterium w* shares antigens with *M. leprae* as well as *M. tuberculosis* suggesting its application in treatment of drug resistant tuberculosis. A randomized control study has demonstrated that this may be responsible for overall reduction of duration of therapy, with no change in sputum conversion rate compared with the traditional short course chemotherapy in new as well as re-treatment cases of tuberculosis (*30,31*). Another advantage though not proven, may be that the Immuvac effect would be longer lasting and could take care of defaulters more meaningfully than chemotherapy alone, leading to a reduction in relapse rate and the emergence of MDR-TB. A meta-analysis to evaluate Mycobacterium w immunotherapy as an adjunct to chemotherapy in participants with pulmonary tuberculosis showed immunotherapy was effective at reducing time to sputum conversion at day 15 and day 30. After day 30, benefit was only demonstrated in the category II TB (re-treatment) (*32*). In a double blind, placebo-controlled trial, pulmonary multidrug-resistant tuberculosis subjects were randomly assigned to receive metronidazole (500 mg thrice daily) or placebo for 8 weeks in addition to an individualized background regimen. More subjects in the metronidazole arm converted their sputum smear ($p = 0.04$) and liquid culture ($p = 0.04$) to negative at 1 month, but these differences were lost by 2 months. Overall, 81% showed clinical success 6 months after stopping therapy, with no differences by arm. Newer nitroimidazoles with both aerobic and anaerobic activity, now in clinical trials, may increase the sterilizing potency of future treatment regimens (*33*).

14. Gene therapy

The decoding of the human genome provides another fascinating aspect in the future therapeutic intervention of tuberculosis. By identifying resistance genes, it will be possible to detect drug resistance before start of therapy and also to develop drugs that target these specific genes, enabling us to considerably reduce the duration of therapy (*34*).

15. Role of steroids

The adjuvant use of corticosteroids in DR-TB patients has been shown not to increase mortality and can be beneficial in conditions such as severe respiratory insufficiency, severe drug induced rashes and central nervous system or pericardial involvement. Prednisolone is commonly used, starting at approximately 1 mg/kg and gradually decreasing the dose to 10 mg per week when a long course is indicated. Corticosteroids may also alleviate symptoms in patients with an exacerbation of obstructive pulmonary disease or when patient is in a very low general condition. In these cases, prednisone may be given in a short course, tapering over 1 to 2 weeks, starting at approximately 1 mg/kg and decreasing the dose by 5 to 10 mg per day. Injectable corticosteroids are often used initially when a more immediate response is needed (*11*). Corticosteroids should never be given to patients with MDR-TB unless they are receiving adequate anti-tuberculosis therapy.

16. Outcome of treatment

The outcome of treatment of MDR-TB is not very favorable and varied from 50-80% in different studies. In a retrospective analysis of 171 immunocompetent patients treated over a ten year period (1973-83) at the National Jewish Hospital in Denver, the overall favorable outcome was only a little over 50% (*35*). All patients were treated with individually tailored regimens in which they received at least 3 or 4 drugs that they had not received previously, or to which they were known to be susceptible. Of the 134 patients evaluated for efficacy, 65% became culture negative and 35% failed to respond. Of those who became culture negative, 14% eventually relapsed giving an overall favorable outcome in 56% of patients. Of the patients who failed, 46% died of tuberculosis. In Cape Province South Africa the 5-year outcome of 240 MDR-TB patients was death in 48%, cure in 33%, 15% were respiratory disabled and 13% were still bacteriologically positive (*36*). However, not all the reports are so grim. In a retrospective analysis reported from South Korea, of 107 patients with MDR-TB treated with at least four drugs to which they had not been exposed to before, or to which they were known to be susceptible, in 63 patients with sufficient follow up data, 52 (82.5%) responded to chemotherapy. There was no subsequent relapse among the patients who responded, and there were no tuberculosis related deaths. The author concluded that, MDR-TB responds relatively well to carefully selected regimens. However, the mean period of follow up was only 17 months (*37*). Khanna, *et al*. (*38*) used kanamycin (6 months), cycloserine, ethionamide and sodium PAS for 18 months in hospitalized patients and observed bacteriological conversion in 71% of MDR-TB patients. Purohit *et a*l. (*39*) used kanamycin (6 months), ethionamide, sodium PAS and isoniazid for 18 months on a domiciliary basis and bacteriological conversion was achieved in 73% of the patients. In another study by Rupak Singla

et al. (*40*) bacteriological quiescence was achieved in 78% of the patients who completed the full therapy. Prasad, *et al*. (*3,41*) observed 75.5% sputum smear and culture conversion rate at the end of two years in 45 patients of MDR-TB patients by using kanamycin, ethionamide, PAS and cycloserine regimen. Out of 45 patients 34 (75.5%) patients were declared cured, these patients were followed for an average 17.4 months (3-60 months) two patients relapsed (5.7%) so long term outcome was around 70%. A systematic review and meta-analysis of the available therapeutic studies on the treatment of multidrug-resistant tuberculosis showed that the overall treatment success estimate was 62%, however, the heterogeneity in study characteristics led to significant variation in reported treatment outcomes (*42*). Individualized treatment regimens had higher treatment success (64%, 95% CI 59-68%) than standardized regimens (54%, 95% CI 43-68%), although the difference was not significant. The proportion of patients treated successfully improved when treatment duration was at least 18 months, and if patients received directly observed therapy throughout treatment. Studies that combined both factors had significantly higher pooled success proportions (69%, 95% CI 64-73%) than other studies of treatment outcomes (58%, 95% CI 52-64%). In another prospective study by Prasad *et al*. of 98 MDR-TB patients treated as per DOTS PLUS protocol it was observed that the default and expiry rates were 7.1% and 10.2% respectively (*43*). It is observed that inadequate treatment duration will result in relapses, may lead to treatment failure and additional acquired drug resistance (*44*). In a study by Chan *et al*. on treatment and outcome analysis of 205 patients with MDR-TB, it was observed that surgical resection and fluoroquinolone therapy was associated with improved microbiological and clinical outcome (*45*). Flouroquinolone resistance was associated with a higher proportion of treatment failures and deaths in MDR patients than non-flouroquinolone-resistant cases. Addition of fluoroquinolones was associated with less chance of relapse and improved treatment outcomes (*46-51*). Kanamycin (*52*), capreomycin (*53*) and streptomycin resistance is associated with poor treatment outcomes. The outcome for patients with MDR-TB is not very favorable in non- immunocompetent patients; and the response in HIV positive patients is even bleaker. In the outbreaks of MDR-TB in and around New York City, between 1988 and 1992, predominantly in HIV infected individuals, mortality was in the order of 80% and the mean duration from diagnosis to death was 4 to 16 weeks. In patients having concomitant HIV and MDR-TB response rate appeared to be poor with high rates of treatment failure and mortality (*54*). In MDR-TB patients with localized disease, surgery, as an adjuvant to chemotherapy, can improve outcomes and should be considered when there is poor response to appropriate chemotherapy (*55*).

17. Conclusion

Multidrug resistant tuberculosis is a growing concern. Prompt identification in early stages and timely initiation of treatment is of utmost importance in curbing further spread of this deadly phenomenon. Despite growing evidence in treatment the outcome still remains unfavorable. It is prudent to formulate management strategies taking into consideration the limitations in diagnostic facilities in developing countries including India. And finally a proper understanding of these management principles is essential for better utilization of available resources, especially in those countries.

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Original Article

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Efficiency of dinoprostone insert for cervical ripening and induction of labor in women of full-term pregnancy compared with dinoprostone gel: A meta-analysis

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Summary **The aim of this study is to evaluate the efficiency of dinoprostone insert, compared with dinoprostone gel, for cervical ripening and induction of labor in women at term. We searched electronic databases and bibliographies of relevant papers to identify randomized controlled trials (RCTs) reporting dinoprostone insert and gel used for cervical ripening and induction of labor. Fifteen RCTs involving 1779 women were included. Dinoprostone insert could greatly contribute to vaginal delivery (VD) within 24 h compared with dinoprostone gel (OR = 2.35, 95% CI = 1.34, 4.13) and the researchers found obvious statistically significant difference (p = 0.003). Yet a meta-analysis of the rates of VD, artificial assisted vaginal delivery and caesarean section (CS) revealed no margin between dinoprostone insert and gel. Dinoprostone insert showed a distinct superiority in terms of VD within 24 h and had an advantage of a shorter hospital stay and less postpartum hemorrhage in contrast to gel. Even though the insert did not perform much better than gel in decreasing the rate of CS and increasing the rate of VD, yet the superior benefit of the vaginal insert compared to gel was still not difficult to observe.**

Keywords: Cervical ripening, caesarean section, dinoprostone, induction of labor, vaginal delivery

1. Introduction

Induction of labor, a common practice that is used in pregnant women, accounts for 20% of all births (*1*). It is applied for the intentional initiation of labor before spontaneous onset, for the purpose of delivery of the fetoplacental unit (*2*). The rate of induction varies by location and is currently more than 20% in America (*3,4*). When the cervix is unfavorable, in order to increase the likelihood of successful induction, promoting cervical ripening is automatically recommended.

Conventionally, oxytocin is used for augmentation of labor in patients with a favorable cervix. Yet for patients with an unfavorable cervix, a sharply ripening agent may be considered. As is well known to all, prostaglandin works efficiently in cervical ripening and labor induction. So dinoprostone surely performs quite well in promoting cervical ripening and labor induction since its main component is prostaglandin E2 (PGE2).

Dinoprostone gel has been successfully used for many years to achieve cervical ripening and induction of labor in women of full-term pregnancy (not less than 37 weeks of gestation) with an unfavorable cervix (Bishop's score < 7). While dinoprostone insert which has also been proved to be effective for cervical ripening and gradual onset of labor for women of full-term pregnancy with suitable indications as a local application through the consistently controlled release of 0.3mg of dinoprostone per hour (*5*). There have been several meta-analyses and systematic reviews evaluating the use of PGE2 and suggesting that it is effective for cervical ripening and labor induction, without distinguishing between dinoprostone insert and gel (*6-8*). A study reported that slow-release PGE2

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vaginal insert achieved cervical ripening and subsequent delivery over a shorter time period (*9*). Conversely, another study declared PGE2 vaginal gel was superior for the induction of labor (*10*). As mentioned above, when it comes to which formulation is optimal, there is an extremely fierce ongoing debate on the preparations of PGE2.

So we assumed that a meta-analysis of published randomized control trails (RCTs) may be beneficial. Thus our objective was chiefly to evaluate the efficiency of dinoprostone insert, compared with dinoprostone gel, for cervical ripening and induction of labor in women at term with an unfavorable cervix and intact membranes.

2. Materials and Methods

2.1. *Search strategy*

We searched PubMed, Medline, the Cochrane Library and bibliographies of relevant papers for articles in English published up to December 2014, using the keywords and combinations of the following search terms "induction of labour/ labor" or "cervical ripening", "intracervical insert" or "Propess" or "Cervidil" or "vaginal pessary" or "dinoprostone insert", "intracervical gel" or "Prepidil" or "dinoprostone gel".

2.2. *Study selection criteria*

We identified RCTs of women of full term pregnancy (not less than 37 weeks of gestation), with intact membrane and unfavorable cervix. Simultaneously, their Bishop's score was less than 7. Dinoprostone insert and gel were given separately to women in treatment group and matched group.

2.3. *Study exclusion criteria*

Abstracts, reviews and unpublished work were excluded because of the absence of details concerning study methods and results. Studies were surely ineligible if there was no information provided on any of the outcomes of focus, if data were not reported regarding the intention to deal with it or if more than 20% of women in either group were lost to follow up.

2.4. *Data extraction, synthesis and analysis*

If the abstract described a study which did not meet the eligible criteria, the study was not reviewed any further. Eligible articles were reviewed in detail. The review of articles was undertaken independently by two reviewers (Zeng and Zhang) who decided which articles were eligible. Any disagreements were resolved by discussing with a third reviewer (Tian).

The two reviewers extracted data for outcomes independently. The primary outcomes were the rates of vaginal delivery (VD) and caesarean section (CS). While VD within 24 h, artificial assisted vaginal delivery, as well as reasons for CS, such as fetal distress, abnormal labor and failure of induction were considered as the secondary outcomes. A subgroup analysis for nulliparous and multiparous women was also conducted. Usage of oxytocin, hospital stay and uterine hyperstimulation were analyzed if data were provided. Baseline data were depicted explicitly if possible.

Statistical analyses were conducted using the program "Review Manager 5.2". We calculated a summary odds ratio (OR) and 95% confidence interval (CI) for dichotomous variables, using Mantel-Haenszel and fixed/random effects mode (*11*). The OR was calculated as the ratio of the number of events using the vaginal insert over that using gel. If the 95% CI did not encompass 1.0 for OR or if the *p* value was less than 0.05, then the results were considered to be statistically significant. Homogeneity of tests among pooled results were performed using simple chi-square test. Quality assessment of the trials was conducted based on the criteria of the Cochrane Handbook for Systematic Reviews of Interventions by categorizing as adequate, inadequate or unclear with respect to allocation concealment, blinding, completeness of follow-up and whether a study was multicenter (*12*). Available from www.cochrane-handbook.org.roach to examine publication bias (*13*).

3. Results

The research generated 53 pieces of paper totally. However, 24 articles were excluded undoubtedly owing to lack of eligible criteria and the remaining 29 articles were reviewed carefully. Among these, 14 articles were not recruited for the following reasons: 5 studies were in abstract only (*14-18*); 5 studies were reviews (*6,19-22*); 3 studies had unavailable data (*23-25*); 1 study had incomplete data (*26*). Finally, 15 RCTs (*27-41*) involving 1779 women were included (Figure 1).

The demographics of the included studies and the methods used for randomization are exhibited (Table 1). The largest number of objectives was 320, while the number of remaining studies enrolled was fewer than 150. Most studies included a large proportion of nulliparous women who accounted for almost more than 60 percent in 9 studies. The basal Bishop score (BBS) in 13 trials was less than 5, with one untold and another ≤ 7. The gestational age (GA) in 14 trails was more than 38 weeks except one was almost 36 weeks. All trials described randomized assignment, usually using computer-generated random numbers, with one trial using opaque, sealed envelopes and another using pre-packed, identical, sealed envelopes to attempt

allocation concealment.

As to quality assessment (Table 2), quality was quite poor on the whole. Only the follow-up achieved total adequateness. Binding was applied in just three studies. Only one had a multi-center study and allocation concealment was inadequate in almost all studies.

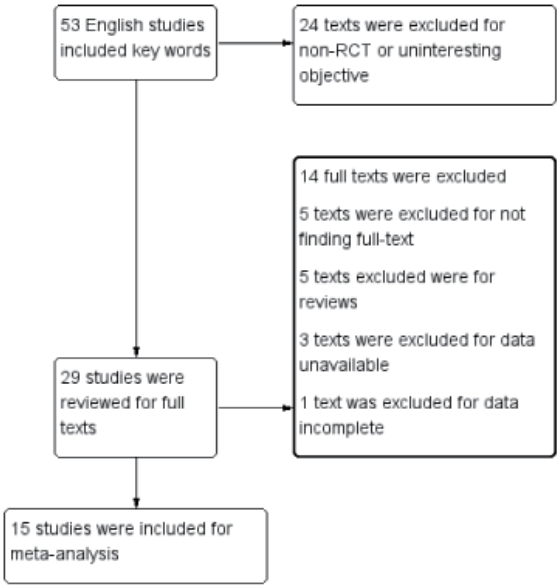

Figure 1. Search algorithm.

Dinoprostone insert could neither increase the odds of VD nor that of artificial assisted vaginal delivery compared with dinoprostone gel (OR = 1.12, 0.96) and there were no statistically significant differences (p = 0.34, 0.87) (Figures 2 and 3). Simultaneously, in contrast to dinoprostone gel, dinoprostone insert cannot decrease the rate of CS (OR = 0.89; 95% CI = 0.71, 1.12) and no statistically significant difference was observed (p = 0.34) (Figure 4). In terms of sub-analysis of reasons for CS, dinoprostone insert could not sharply decrease the rate of CS owing to fetal distress, abnormal labor and failure of induction, compared to dinoprostone gel. Gratefully, dinoprostone insert did much better in contributing to VD within 24 h than dinoprostone gel (OR = 2.35, 95% CI = 1.34, 4.13) and the researchers found an obvious statistically significant difference (p = 0.003) (Figure 5). To evaluate possible exiting publication bias for the outcome, a funnel plot demonstrated no evidence of asymmetry, suggesting that publication bias was not present (Figure 6).

4. Discussion

The findings indicate that dinoprostone insert could increase the rate of VD within 24 h, which was consistent with the conclusion of a current study (*42*). While the

Table 1. Description of populations and methods of randomization of included studies

Study	Country	NP, *n* (%)	MA (years)	BBS	GA (weeks)	Method of randomisation
Vollebregt *et al.*	Netherlands	33 (66)	30.4 ± 4.2	–	40.3 ± 1.8	–
		37 (74)	29.9 ± 4.9	–	40.5 ± 1.9	–
D'Aniellol *et al.*	Italy	54 (82)	29.3 ± 3.2	3.64 ± 1.43	40.5 ± 2.7	Computer-generated
		37 (75)	28.6 ± 3.7	3.5 ± 1.04	40.9 ± 2.7	as assignment
Marconi *et al.*	Italy	109 (66.7)	30.3 ± 5.1	4.0 ± 1.1	39.6 ± 1.4	Computer-generated
		111 (68.9)	31.0 ± 4.7	4.1 ± 1.2	39.6 ± 1.3	as assignment
Facchinetti1 *et al.*	Italy	70 (100)	29.1 ± 4.9	≤ 3	40.7 ± 1.4	Computer-generated
		70 (100)	27.9 ± 5.1	≤ 3	40.9 ± 1.1	as assignment
Ramsey *et al.*	Ireland	22 (57.9)	26.7 ± 3.6	3.0 ± 1.2	39.3 ± 1.3	Computer-generated table
		21 (60.0)	28.0 ± 4.4	3.0 ± 1.2	39.2 ± 1.3	with opaque,sealed envelopes
Grignaffini *et al.*	Italy	36 (71)	30.0 ± 3.6	< 5	40 ± 1.0	Unclear
		40 (77)	31.0 ± 4.9	< 5	40 ± 1.0	
Strobelt *et al.*	Italy	34 (61)	33*	≤ 4	37-41	Single-blind
		30 (59)	33*	≤ 4	37-41	randomisation
Connell *et al.*	UK	–	27.6	3.8	40.4	Computer-generated
		–	28.1	3.6	40.4	as assignment
Kalkat *et al.*	UK	13(43)	27.1 ± 5.4	3.5 ± 1.4	38.5 ± 1.9	Pre-packed, identical,
		10(33)	27.1 ± 5.9	3.3 ± 1.6	38.8 ± 1.7	sealed envelopes.
Ottinger *et al.*	America	28(62)	26.0 ± 7.0	2.8 ± 1.6	39.1 ± 3.0	Computer-generated
		27(60)	25.2 ± 6.7	2.9 ± 1.9	39.0 ± 2.8	as assignment
Chyu *et al.*	Chicago	20(54)	29.8 ± 5.7	≤ 7	39.2 ± 2.1	Computer-generated
		18(50)	27.8 ± 6.5	≤ 7	39.0 ± 2.2	as assignment
Stewart *et al.*	Oklahoma, USA	39(53)	24.8 ± 5.8	3.4 ± 1.5	39.9 ± 1.6	Computer-generated
		38(49)	24.1 ± 6.1	3.5 ± 1.4	40.1 ± 1.5	as assignment
Hennessey *et al.*	Oklahoma, USA	15(42)	23.4*	1.6 ± 0.7	36.9 ± 3.4	Computer-generated
		16(47)	24.4*	1.9 ± 0.5	35.9 ± 3.6	as assignment
Facchinetti *et al.*	Italy	58(100)	29.7 ± 4.8	< 4	41.7 ± 0.6	Computer-generated
		58(100)	29.1 ± 5.9	< 4	41.6 ± 0.8	as assignment
Triglia *et al.*	Italy	52(80)	31*	2	41.0 ± 3.0	Computer-generated
		46(71)	32*	3	41.0 ± 3.0	as assignment

BBS, basal Bishop score; MA, maternal age; NP, nulliparous; GA, gestational age; *, mean; —, data unavailable. Values are mean ± S.D.

Table 2. Description of quality assessment of included studies

Study	Treatment	Comparision	Allocation concealment	Blinding	Follow-up	Multicenter
Vollebregt *et al*.2002	Controlled release PgE2 (Propess)PV 10 mg (12h), *n* = 50	PgE2 gel (Prepidil) 0.5 mg IC one dose, *n* = 50	IA	IA	A	IA
D'Aniellol *et al*.2003	PgE2 pessary 10 mg (12 h), *n* = 49	PgE2 gel 0.5 mg IC one dose, *n* = 66	IA	IA	A	IA
Marconi *et al*.2008	Controlled release PgE2 (Propess)PV 10 mg (12 h), *n* = 159	PgE2 gel (Prepidil) 0.5 mg IC or 1 mg IV 6 hours up to three doses, *n* = 161	IA	IA	A	IA
Facchinetti *et al*.2005	Controlled release PgE2 (Propess)PV 10 mg (12 h), *n* = 70	PgE2 gel (Prepidil) 0.5 mg IC Q12 h, *n* = 70	IA	A	A	IA
Ramsey *et al*.2003	Controlled release PgE2 (Cervidil)PV 10 mg (12 h), *n* = 38	PgE2 gel (Prepidil) 0.5 mg, 6 hours up to two doses, *n* = 35	IA	IA	A	IA
Grignaffini *et al*.2004	Slow release PgE2 (Propess) PV 10 mg (12 h), *n* = 51	PgE2 gel (Prepidil) 1.0 mg, 6 hours up to two doses, *n* = 52	U	U	A	IA
Strobelt *et al*.2006	Slow release PgE2 (Propess) PV 10 mg (12 h), *n* = 56	PgE2 gel (Prepidil) 0.5 mg, 6 hours up to two doses, *n* = 51	IA	A	A	A
Connell *et al*.2006	Sustained-release PgE2 (Propess)PV 10 mg (12 h), *n* = 34	PgE2 gel (Prostin) 0.5 mg, 6 hours up to two doses, *n* = 38	IA	IA	A	IA
Kalkat *et al*.2008	Slow release PgE2 (Propess) PV 10 mg (12 h), *n* = 60	PgE2 gel (Prostin), unclear, *n* = 60	A	A	A	IA
Ottinger *et al*.1998	Controlled release PgE2 (Cervidil)PV 10 mg (12 h), *n* = 45	PgE2 gel (Prepidil) 0.5 mg,6 hours up to two doses, *n* = 45	IA	IA	A	IA
Chyu *et al*.1997	Controlled release PgE2 (Cervidil)PV 10 mg (12 h), *n* = 37	PgE2 gel (Prepidil) 0.5 mg, unclear, *n* = 36	IA	IA	A	IA
Stewart *et al*.1998	Controlled release PgE2 (Cervidil)PV 10 mg (12 h), *n* = 37	PgE2 gel (Prepidil) 0.5 mg, unclear, *n* = 36	IA	IA	A	IA
Hennessey *et al*.1998	Controlled release PgE2 (Cervidil)PV 10 mg (12 h), *n* = 36	PgE2 gel (Prepidil) 0.5 mg, unclear, *n* = 34	IA	IA	A	IA
Facchinetti *et al*.2007	Slow release PgE2 PV 10 mg (12 h), *n* = 58	PgE2 gel, unclear, *n* = 58	IA	IA	A	IA
Triglia *et al*.2010	(Pessary)PV 10 mg (24 h), *n* = 65 Controlled release PgE2	PgE2 gel (Prepidil), 2.0 mg up to two doses, *n* = 65	IA	U	A	IA

Mg, microgram; PV, per vaginum; Q, every; h, hour; IC, intracervical; IV, intravaginal; A, adequate; IA, inadequate; U,unclear.

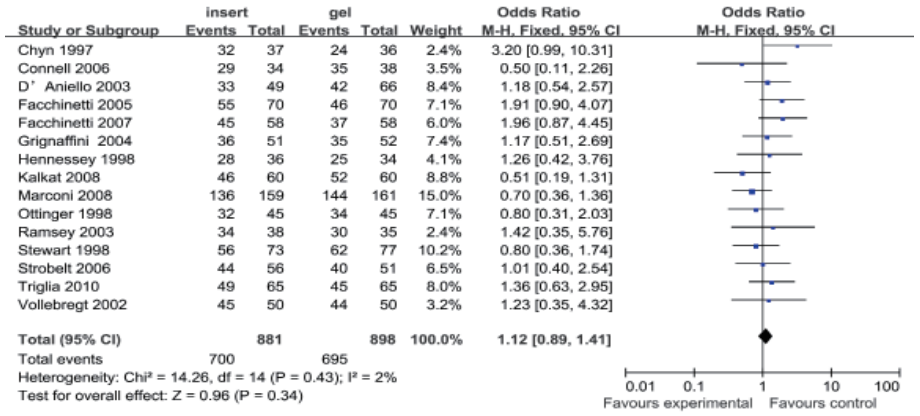

Figure 2. Meta-analysis of data about vaginal delivery (VD) from 15 studies using a fixed-effect model. CI, confidence interval; OR, odds ratio.

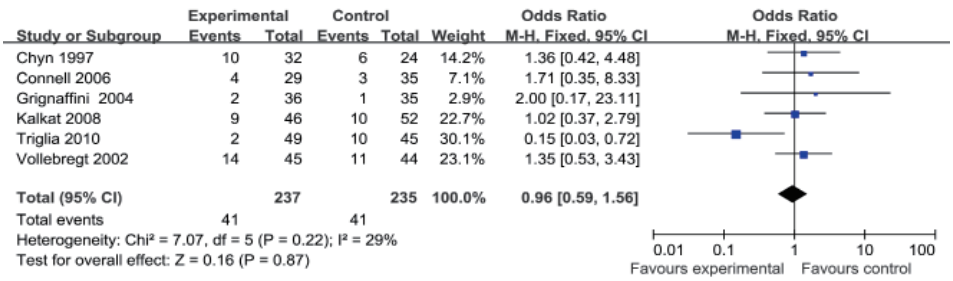

Figure 3. Meta-analysis of data about artificial assisted vaginal delivery from 5 studies using a fixed-effect model. CI, confidence interval; OR, odds ratio.

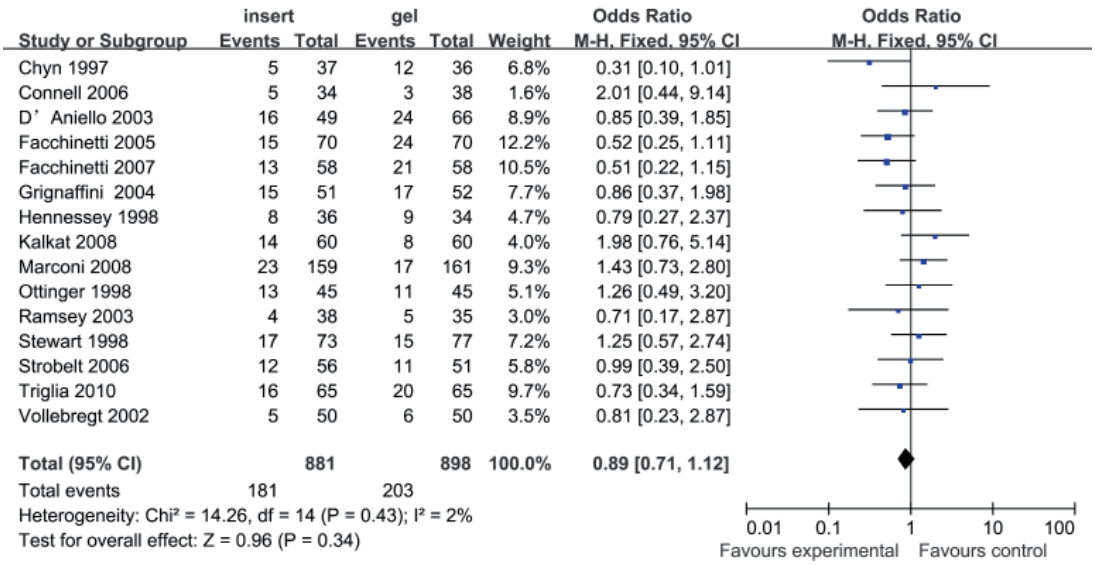

Figure 4. Meta-analysis of data about caesarean section (CS) from 15 studies using a fixed-effect model. CI, confidence interval; OR, odds ratio.

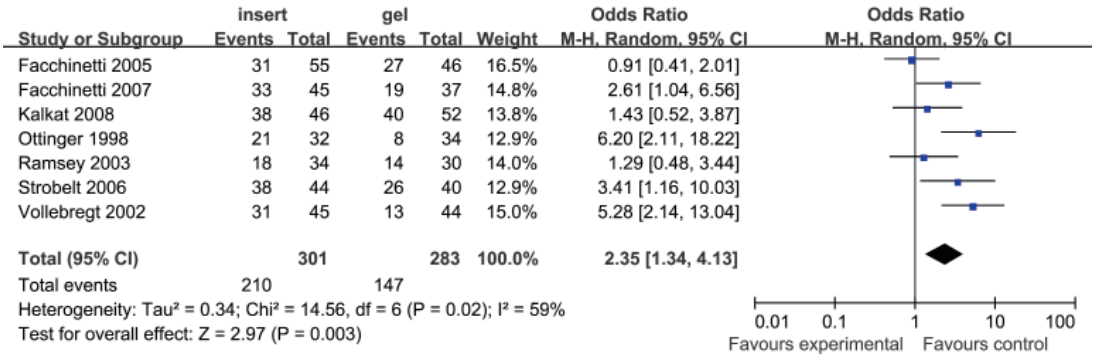

Figure 5. Meta-analysis of data about vaginal delivery within 24 h from 7 studies using a random-effect model. CI, confidence interval; OR, odds ratio.

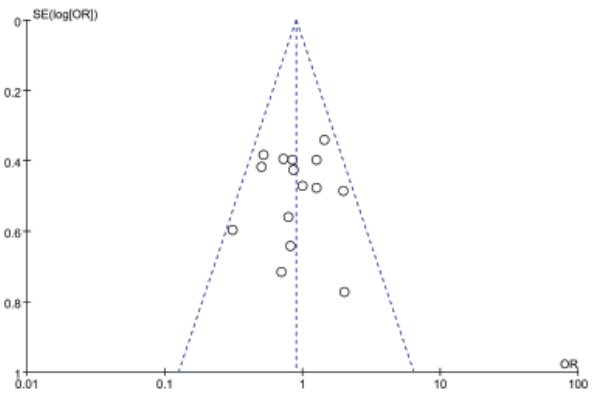

Figure 6. Publication bias is assessed by funnel plot, of which the asymmetry is exhibited by evidence of small studies with higher odds ratio and the paucity of small negative studies in the lower right of the funnel plot.

insert did not appear to be more effective than gel in altering the rates of VD, CS, or artificial assisted vaginal delivery, as well as the reasons for CS. This distinguished with a previous report which showed dinoprostone insert had a better effect than gel (*26*). The inconspicuous advantage may attribute to that the vaginal insert was applied for only 12 h in most cases and we probably speculated that a longer application of insert, such as 24 h, may generate a remarkable difference.

In order to test whether a distinction exists between nullipara and multipara, researchers conducted an analysis of a subgroup of VD and CS for dinoprostone insert and gel in nullipara and multipara cases respectively. Results showed both insert and gel assisted more in promoting VD and decreasing the odds of CS in multipara than nullipara. By comparing the ORs, insert appeared to contribute much more than gel. However, the net effect of dinoprostone was worth further investigation in that the possibilities of successful cervical ripening and induction of labor were intrinsically greater in multipara.

Oxytocin was utilized additionally during the process of induction of labor as well, so researchers also gave a glimpse of situations where it occurred in 5 studies (*28,30,32,33,40*). Concerning its homogeneity,

the fixed model was used to achieve the aim (OR = 0.65, 95% CI = 0.35, 1.20). The OR appeared to convey that the utilization rate of oxytocin in the insert group was lower, but no statistically significant difference was observed (p = 0.17). This was inconsistent with previous reports which assumed less need for oxytocin was necessary in the process with the insert working (*43,44*).

The length of hospitalization was reported in only 2 studies (*32,33*), evaluating 246 women, with 123 receiving dinoprostone insert. The result revealed the rates of hospital stay more than 4 days were 0.41 *vs*. 0.46 with respect to dinoprostone insert and gel. Also, postpartum hemorrhage was reported in 2 studies (*31,32*) showing that the rates were 0.13 versus 0.23 regarding dinoprostone insert and gel separately. There existed a flaw that the definition of postpartum hemorrhage in the two studies were not given clearly. In terms of uterine hyperstimulation, Strobelt and D'Anie demonstrated the rates between the two were 0.034 and 0.020 (*28,31*). To conclude, the insert had an advantage of shorter hospital stay and less postpartum hemorrhage in contrast to the gel. However, the insert may have a higher rate of uterine hyperstimulation, even if the rate is quite low. That still leaves a significant flaw. Since a recent study comparing the efficacy of 24h vaginal insert of PGE2 in comparison to vaginal gel of PGE2, a similar rate of uterine hyperstimulation was found (*41*).

Nevertheless, dinoprostone insert functions through the consistent, controlled release of 0.3 mg dinoprostone to attain the aim of a gradual onset of cervical ripening and labor induction. Hence, the times of vaginal examination using dinoprostone insert decrease sharply in contrast to gel during the process of labor. Namely, it could help to lessen doctors' workload and patients' discomfort arising from vaginal examination. From this point of view, dinoprostone insert is surely superior to gel.

In addition, we failed to appraise the risks of Apgar score less than 7 at 5 minutes, admission to a neonatal intensive care unit (NICU), perinatal mortality, neonatal morbidity (such as birth asphyxia and neonatal encephalopathy), maternal morbidity (such as chorioamnionitis, sepsis, uterine rupture and admission to an intensive care unit), maternal mortality and adverse effects (such as nausea, vomiting, and diarrhea) on account of data unavailable.

Moreover, the absence of adequate data about the related risks, such as length of hospitalization, postpartum hemorrhage and hyperstimulation, leads to an observation that analysis of safety was greatly contracted. Although a study reported that sustained-release of dinoprostone led to spontaneous induction of labor without increasing the obstetrical risks in a majority of patients (*45*) but further investigation about this issue should be conducted in the future.

The limitation of this meta-analysis, from the methodological aspect, is that the included studies are mostly of low-to-medium quality. Although we searched all of the worldwide literature, the 6 included studies came from Italy, the others were Netherlands, Ireland, England and America. Perhaps the lack of multicenter studies leads to a deviation. Despite all of the shortages above, the strength of our survey is stronger than any single study since the included primary studies are quite homogeneous, and it incorporated 15 RCTs involving 1,779 women.

Given the previously mentioned variability in characteristics of the patients, locations, and methodologies, careful interpretation of the results should be taken into consideration. Totally, dinoprostone insert does yield a distinct superiority in terms of VD within 24 hours and has the advantage of shorter hospital stay and less postpartum hemorrhage in contrast to gel. However, the insert does not perform much better than gel in decreasing rates of CS and promotion of VD in women at term with intact membrane and an unfavorable cervix. There is a consideration that the insert may have a higher rate of uterine hyperstimulation, even if the rate is quite low. Even so, the superior benefit of vaginal insert compared to gel can be easily seen.

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Original Article

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Topical administration of tranexamic acid in total hip arthroplasty: A meta-analysis of Randomized Controlled Trials

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Summary **Tranexamic acid (TXA) is an antifibrinolytic drug which has been widely used in many areas of surgery. The purpose of our meta-analysis was to review randomized controlled trials (RCT) of the effectiveness and safety of topical TXA treatment in reducing total blood loss and transfusion rate for patients undergoing primary total hip arthroplasty (THA). A literature search was undertaken. Five eligible reports match the inclusion and exclusion standard. The topical administration of TXA groups revealed lower total blood loss(mean difference - 347ml, 95% CI, - 411 to - 282 mL; $p < 0.00001$) and transfusion rate (OR, 0.23 ($p < 0.00001$; 95% CI, 0.14-0.38)) compared with control groups. Meanwhile there was no statistically significant difference in the risk of developing thromboembolic events (OR, 1.64 (95% CI, 0.39-6.97); $p = 0.5$). Topical administration of TXA could significantly reduce total blood loss and transfusion requirements in primary THA, without increased thromboembolic complications.**

Keywords: Topical administration, tranexamic acid, total hip arthroplasty, intra-articular, meta-analysis

1. Introduction

Total hip arthroplasty (THA) is an effective treatment for osteoarthritis of the hip, femoral neck fracture, aseptic necrosis of femoral head and congenital developmental dysplasia of the hip. However, THA is associated with considerable perioperative blood loss and subsequently requires allogenic transfusion (*1,2*). Such allogenic transfusions may carry potential hazards of adverse immunological reactions, intravascular hemolysis, transfusion-related acute lung injury, disease transmission, transfusion-induced coagulopathy, renal failure, and even increased mortality (*3-5*).

Tranexamic acid (TXA), a synthetic derivative of lysine, prevents fibrinolysis by reversible blockade of the lysine-binding sites of plasminogen, thereby achieving the goal of local hemostasis and reducing bleeding (*6,7*). Currently, TXA has been widely used in many areas of surgery, such as, cardiovascular surgery, gastrointestinal bleeding, postpartum hemorrhage, orthotopic liver transplantation, and hip and knee arthroplasty (*8-11*). Intravenous administration of TXA has been reported to reduce blood loss in primary THA (*12-14*). However, one significant concern with TXA, is the risk of systemic thrombotic events which have been taken into consideration.

In consideration of thrombotic events with intravenous administration, more and more scholars have started to transfer their attention to topical use of TXA (*15,16*). Compared with IV-TXA, it has been hypothesised that topical application provides maximum concentration of TXA at the bleeding site, and is associated with little or no systemic absorption of TXA. However, clinical efficacy is inconsistent (*17,18*) and an optimal TXA treatment protocol is still unknown. We therefore performed a meta-analysis of randomized controlled trials (RCT) to investigate the effectiveness and safety of topical TXA treatment in reducing total blood loss and transfusion rate for patients undergoing primary THA.

2. Methods

This systematic review and meta-analysis was based on the standards described in Preferred reporting items for systematic reviews and meta-analyses (*19*).

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2.1. *Inclusion and eligibility criteria*

Only randomized controlled trials (RCTs) studies were eligible for this study, the inclusion criteria are as follows: (*i*) patients were adults who received primary unilateral THA regardless of the type or size of prosthesis implanted; (*ii*) the intervention was topical(intra-articular)administration of TXA; (*iii*) the full text of each article was available; (*iv*) outcome measures included total blood loss, transfusion rate, and incidence of thromboembolic complications. Exclusion criteria: (*i*) patients who had allergy to tranexamic acid; (*ii*) thrombotic disorder; (*iii*) patients who were on anticoagulant treatment.

2.2. *Search strategy*

Two independent reviewers searched the PubMed, Elsevier, Ebsco host, and OVId, to acquire all relevant articles. There were no restrictions as to the language and date. The key words used for the search included: "tranexamic acid" or "cyklokapron" and "total hip replacement" or "total hip arthroplasty" and "topical" or "intra-articular". The reference lists of related reviews and meta-analyses were reviewed for any potential studies.

2.3. *Extraction of data*

Each date extraction was reviewed and agreed upon by the authors, and a preliminary test was performed to ensure its consistency. The complete manuscript was obtained and carefully scrutinized by the two reviewers. Any disagreement between them was resolved by consensus or consultation with the senior authors. Data extracted included: author's name, publication year, sample size, dose of TXA, method of TXA administration, type of control, postoperative thromboprophylaxis, THA prothesis, anesthesia, DVT screening method, total blood loss, transfusion rate, and incidence of thromboembolic complications.

2.4. *Study quality*

The methodological quality of included studies was assessed with the use of Jadad Score by two independent reviewers (*20*). Any disagreement was resolved by consensus or the senior authors. Studies with a Jadad score of 1 were considered poor, scores of 2 were considered adequate and a score of 3 or higher was considered as high quality.

2.5. *Statistical analysis*

The meta-analysis program of the Review Manager Database (RevMan version 5.2, Copenhagen, The Nordic Cochrane Centre, The Cochrane Collaboration, 2012.) was utilized to analyze selected data. Continuous data were summarized using the weighted mean differences and respective 95% confidence intervals (95% CI). Dichotomous data were summarized using odds ratio (OR) and 95% confidence intervals (95% CI). The presence of heterogeneity was assessed using Chi square test and I square test. A p value < 0.1 and I_2 > 50% were considered presence of statistical heterogeneity. A random-effects model analysis was used to estimate trials showing heterogeneity while a fixed-effect model analysis was used for the reverse. A non-significant test result(a p value > 0.1 and an I_2 value < 50%) only suggested that there was no evidence of heterogeneity; it did not necessarily signify homogeneity, as there may have been insufficient power to detect heterogeneity.

3. Results

A total of 277 abstracts and titles were reviewed. After screening, eventually five (*21-25*) eligible reports matched the inclusion and exclusion standard for analysis (Figure1). Two studies (*21,22*) included topical administration of TXA in total hip arthroplasty and total knee arthroplasty. One study (*25*) included two experimental groups (intravenous injection of TXA and topical administration of TXA). Only outcome

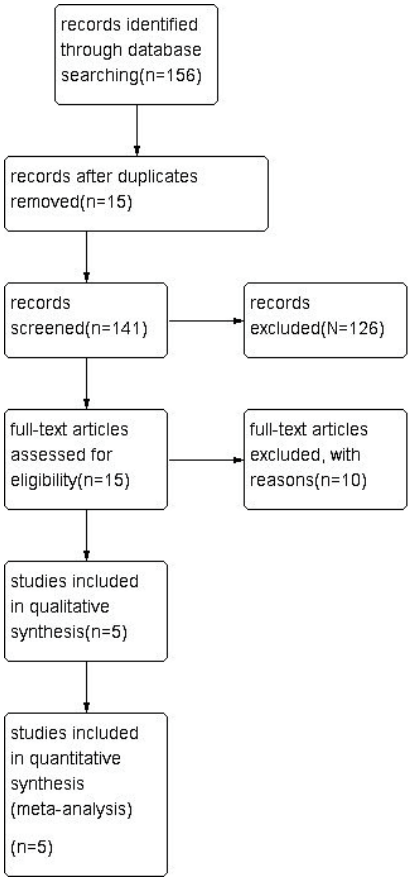

Figure 1. Flowchart of the study selection.

Table 1. Characteristics of included studies

Author	Number (TXA/Con)	Intervention (TXA)	Con	Thromboprophylaxis	Bone cement	Anesthesia	DVT screen
Konig (2013)	91/40	3gTXA/100mlNS topically used at three points during the procedure	None	Not mentioned	Cementless	Spinal	Not mentioned
Martin (2014)	25/25	2gTXA/100mlNS inject into the joint prior to surgical closure	100mlNS	Warfarin or aspirin	Cemented (15) cementless (35)	General or spinal	Clinical
Yue (2014)	52/49	3gTXA/150mlNS topically used 150mlNS at three points during the procedure	150mlNS	LMWH	Cementless	General or spinal	Ultrasound
Alshryda (2013)	81/80	Topical (intra-articular) application of tranexamic acid	Not mentioned	Mechanical and mechanical + LMWH when BMI>30 kg/m^2	Cemented (8) cementless (120) hybrid (33)	General or spinal	Doppler ultrasound
Wei (2014)	102/100	3gTXA/100mlNS topically used at three points during the procedure	100mlNS	LMWH	Cementless	Not mentioned	Ultrasound

TXA: tranexamic acid, Con: control, LMWH: low molecular weight heparin, DVT: deep vein thrombosis.

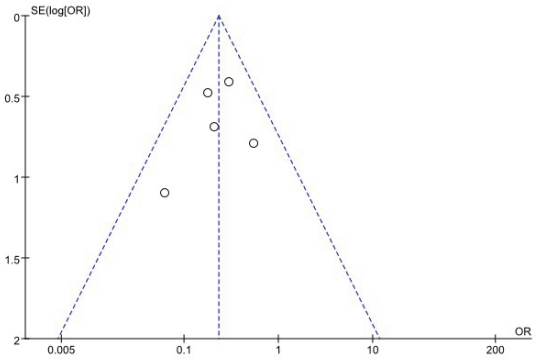

Figure 2. Funnel plot of transfusion rate. SE, standard error.

measurements referring to "THA" and "topical" group were analyzed in the present study. Only one report (*21*) did not mention thromboprophylaxis, and the method for screening thrombosis. The details of the included studies are listed in Table 1.

Figure 2 represents funnel plots examining for potential publication bias between studies. These funnel plots reports the logs OR of the numbers of patients requiring allogeneic transfusions as a measure of the treatment effect, and it shows moderate asymmetry, indicating mild publication bias.

3.1. *Total blood loss*

Four studies including 595 patients reported total blood loss (*21, 23-25*). The combination of data from the studies revealed lower total blood loss in topical administration of TXA groups compared with control groups (mean difference – 347 mL, 95% CI, − 411 to − 282 mL; $p < 0.00001$). Forest plots summarized the meta-analysis including data comparing total blood loss between TXA groups and control groups (Figure 3).

3.2. *Transfusion requirements*

The number of patients who needed allogenetic blood transfusion was recorded in five trials with 654 patients. Transfusion rate was significantly lower in the topical administration TXA group compared with the control group (OR, 0.23 (95% CI,0.14-0.38)) (Figure 4).

3.3. *Thromboembolic complications*

The data of thromboembolic complications was available in all five studies. Three studies (*21,24,25*) reported six thromboembolic events, four of 351 in the TXA group and two of 294 in the control group. However, there was no statistically significant difference in the risk of developing thromboembolic events (OR, 1.64 (95% CI, 0.39-6.97); $p = 0.5$) (Figure 5).

4. Discussion

This meta-analysis only analyzed randomized controlled trials (RCTs) so as to minimize the possibility of bias. Our results showed that topical (intra-articular) administration of TXA could effectively reduce total blood loss, and allogeneic blood transfusion rate. Also, topical (intra-articular) administration of TXA did not demonstrate increased risk of DVT, PE or other adverse events. This is consistent with the meta-analyses by Panteli in total knee replacement (*26*). However, given the relatively short duration of postoperative follow-up, and small sample involved, these findings require further confirmation.

All the five studies were graded with high quality scores, while the methodological quality of included trials was insufficient. Only three trials were almost identical, and one did not mention the placebo group

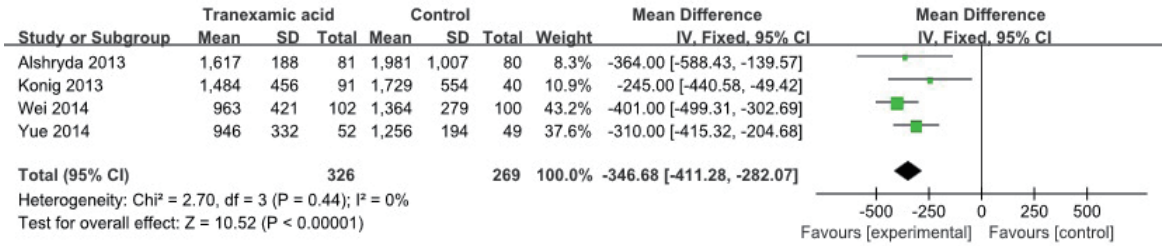

Figure 3. Trials of TXA versus control:forest plot of total blood loss.

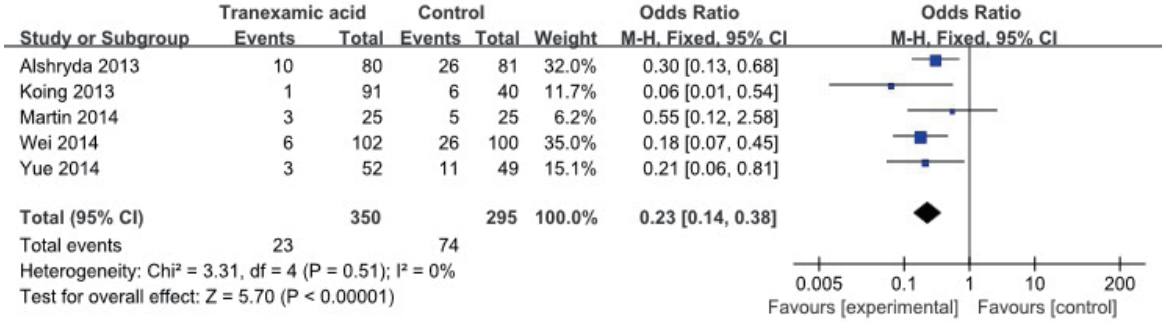

Figure 4. Trials of TXA versus control:forest plot of transfusion rate.

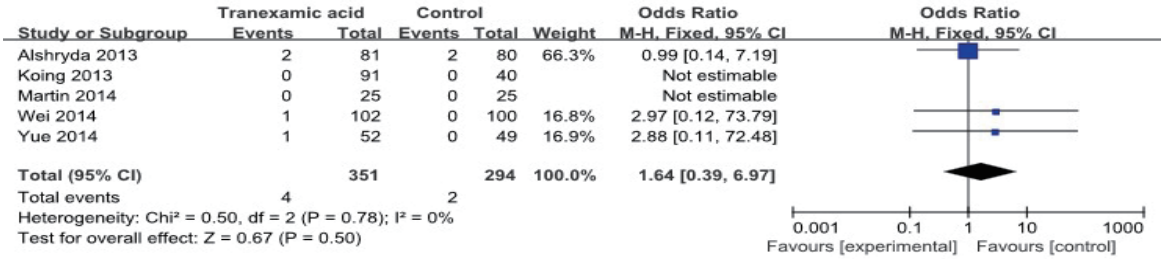

Figure 5. Trials of TXA versus control:forest plot of thromboembolic complications.

(*21*). The remaining two different method trials: one 2 g TXA/100 mL NS injected into the joint prior to surgical closure (*22*), the other topical (intra-articular) application of tranexamic acid (*24*) and did not mention the dose of TXA. These differences could have resulted in biased and flawed results. Furthermore, we believe that patient characteristics variation, postoperative thromboprophylaxis, anesthesia type, dose of TXA, method of TXA administration, and THA prothesis type, can distort the results. Although in this study heterogeneity is very low, it did not necessarily imply homogeneity. There may have been potential heterogeneity, because we lack sufficient power to detect it.

TXA is an antifibrinolytic agent and it has been confirmed that intravenous TXA could effectively reduce bleeding and transfusion rates in THA and TKA (*7,17*). Additional meta-analyses and systematic reviews about the efficacy of intravenous TXA in THA have been published (*27,28*). However, the risk of systemic thrombotic events must be taken into consideration and it is generally agreed that only a small percentage of the intravenously injected drug reaches the target location to inhibit tissue fibrinolysis and stabilize clots (*29*). Compared with IV-TXA, a topical (intra-articular) administration has the advantage of providing a maximum concentration of TXA at the bleeding site, and is associated with little or no systemic absorption of the TXA (*24*). So far,there are few meta-analyses about intra-articular injection of tranexamic acid and the efficacy of topical administration of TXA is obscure in THA and therefore we conducted this study.

The limitations of our study include small sample size (only 654 patients) and insufficient data are available to support the analysis of hip joint function and quality of life. Furthermore, the method of TXA administration, dose of TXA, THA prothesis type, and surgical technique may affect the outcome.

In conclusion, this meta-analysis demonstrated that topical administration of TXA could significantly reduce total blood loss and transfusion requirements in primary THA, without significant increase of thromboembolic complications. Because of the small sample size, more prospective randomized controlled studies with a larger scale of patients are necessary.

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Original Article

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Compounds in a particular production lot of tryptic soy broth inhibit *Staphylococcus aureus* cell growth

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Summary ***Staphylococcus aureus* Newman strain and several methicillin-resistant *S. aureus* (MRSA) clinical isolates were grown on agar plates prepared with conventional lots of tryptic soy broth (TSB). Cell growth of these strains was inhibited on agar plates containing TSB of a particular product lot (lot A), whereas the cell growth of *S. aureus* RN4220 strain and several other MRSA clinical isolates was not inhibited. The cell growth of a strain of *S. epidermidis* was also inhibited on agar plates containing TSB of lot A, whereas the cell growth of *Bacillus subtilis*, *Lactococcus lactis*, *Klebsiella pneumonia*, *Salmonella enterica*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Escherichia coli* was not inhibited. Although cell growth of the Newman strain was inhibited on agar plates containing TSB of lot A that was autoclaved in stainless steel or glass containers, cell growth inhibition was not observed when the medium was autoclaved in polypropylene containers. Compounds that inhibited the cell growth of the Newman strain were extracted from a polypropylene tube that was preincubated with liquid medium prepared from TSB of lot A. These findings suggest that polypropylene-binding compounds in TSB of lot A inhibited the cell growth of *S. aureus* Newman strain, some MRSA clinical isolates, and *S. epidermidis*.**

Keywords: MRSA, polypropylene-binding compounds, growth inhibition

1. Introduction

Staphylococcus aureus infects immunocompromised patients and causes opportunistic diseases such as sepsis and meningitis (*1*). At present, half of the *Staphylococcus aureus* strains isolated from clinics in Japan are resistant to methicillin, a beta-lactam antibiotic. Methicillin-resistant *S. aureus* (MRSA) clinical isolates are resistant to various other antibiotics as well, making it difficult to adequately treat patients infected with MRSA (*2*). To overcome MRSA infection, a comprehensive understanding of the mechanisms of drug resistance and pathogenicity based on genetic and biochemical analyses is necessary. Consistent MRSA culture techniques are essential for these analyses.

Tryptic soy broth (TSB) is routinely used for culturing *S. aureus* clinical isolates. We observed that the Newman strain, a standard strain of *S. aureus*, did not form colonies on agar plates prepared with TSB of a particular product lot (lot A), whereas the RN4220 strain, another standard strain of *S. aureus*, did form colonies on the agar plates prepared with TSB of lot A. Here we describe that polypropylene-binding compounds in TSB of lot A inhibited the cell growth of certain *S. aureus* strains.

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2. Materials and Methods

2.1. *Reagents and containers*

Tryptic soy broth (TSB) was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Product lots of TSB (lot 1291840 [lot A] and lot 1137750 [lot B]) were used. Agar was purchased from Nacalai Tesque (Kyoto, Japan). Polypropylene tubes (50-mL conical tubes) were purchased from Corning Inc. (Corning, NY, USA). Glass conical flasks, glass test tubes, and glass bottles were purchased from Iwaki (Osaka, Japan). A stainless steel kettle (3 L) was purchased from Bestco (Osaka, Japan).

2.2. *Preparation of media*

TSB medium were prepared according to the manufacturer's protocol. Specifically, 30 g TSB powder was dissolved in 1 L water. For preparation of the agar medium, 15 g agar was added to the TSB medium, and the mixture was autoclaved at 121°C for 15 min in the stainless steel kettle, a conical flask, or a polypropylene tube. After autoclaving, the agar medium was poured into 9-cm diameter plastic plates (AS ONE Corporation, Osaka, Japan), cooled, and solidified. In the experiment in which pieces of polypropylene were added to the TSB medium, polypropylene tubes were cut with scissors into 1.5-cm square pieces, and 0.26 g of the pieces were added per 1 mL TSB.

2.3. *Bacteria and growth conditions*

The bacterial strains used in this study are shown in Table 1. In the case of liquid culture, overnight cultures were diluted to 1:100 in TSB liquid medium. The cultures were incubated at 37°C and the turbidities at 600 nm were measured using a spectrophotometer (Shimadzu Corporation). In the case of bacterial culture on agar plates, overnight cultures were appropriately diluted with 0.9% NaCl, then 100 µL of the dilution was spread on a TSB agar plate or streaked on a TSB agar plate using a loop, and the plates were incubated at 37°C.

2.4. *Preparation of acetone extracts of polypropylene-binding compounds in TSB*

Liquid medium prepared with TSB of lot A was autoclaved in a glass bottle, transferred to a polypropylene tube, and incubated for 16 h at room temperature. The liquid was removed by decantation from the tube. Acetone (10 mL) was added and the mixture vortexed. Five milliliters of the acetone extract was transferred to a glass test tube and the acetone was removed by evaporation at 60°C. Liquid media prepared with TSB of another lot (2.5 mL) was added to the dried test tube and used for the experiment.

Table 1. Bacterial strains used in this study

Species	Strains	References
Staphylococcus aureus	Newman	(*6*)
	RN4220	(*7*)
	MRSA3	(*8,9*)
	MRSA4	(*8,9*)
	MRSA5	(*9*)
	MRSA6	(*8,9*)
	MRSA8	(*8,9*)
	MRSA9	(*8,9*)
	MRSA11	(*8,9*)
	MRSA12	(*8,9*)
Staphylococcus epidermidis	ATCC12228	(*10,11*)
Bacillus subtilis	168	(*12*)
Lactococcus lactis	MG1363	(*13*)
	11/19-B1	This study
Enterococcus mundtii	EM1s	This study
Klebsiella pneumonia	ATCC10031	(*14*)
Salmonella typhimurium	ATCC14028s	(*15*)
Serratia marcescens	2170	(*16*)
Pseudomonas aeruginosa	PAO1	(*17*)
Escherichia coli	JM109	(*18*)
	O-157 Sakai	(*19,20*)

3. Results

3.1. *Cell growth inhibition of S. aureus Newman strain on agar plates prepared with TSB of a particular lot*

TSB medium is frequently used to culture *S. aureus*. We observed that *S. aureus* Newman strain did not grow on medium prepared with TSB of a particular lot (lot A). Although Newman strain on agar plates prepared with TSB of another lot did form colonies, it did not form colonies on agar plates prepared with TSB of lot A (Figure 1A). The RN4220 (RN) strain formed colonies on agar plates containing TSB of both lots (Figure 1A). Thus, the cell growth of *S. aureus* RN4220 strain was not inhibited by TSB of lot A.

3.2. *Cell growth inhibition of MRSA strains on agar plates containing TSB of lot A*

We then examined whether MRSA strains grew on agar media prepared with TSB of lot A. All seven strains examined grew on agar plates prepared with TSB of lot B. The cell growth of five strains (MRSA4, MRSA5, MRSA8, MRSA11, and MRSA12) on agar plates prepared with TSB of lot A was markedly inhibited compared to that on plates with TSB of lot B (Figure 1B). Two MRSA strains (MRSA5 and MRSA11), as well as the Newman strain, did not grow at all on agar plates prepared with TSB of lot A. Therefore, cell growth of the majority of MRSA clinical isolates was inhibited in TSB of lot A.

3.3. *Cell growth of other species of bacteria on agar plates containing TSB of lot A*

We compared the cell growth of 11 strains (9 species) of bacteria on agar media prepared with TSB of lot A or lot B. *S. epidermidis* ATCC12228 formed colonies on agar plates prepared with TSB of lot B, it did not form colonies on agar plates prepared with TSB of lot A (Figure 1C). On the other hand, cell growth of *Bacillus subtilis* 168 strain, *Lactococcus lactis* MG1363 strain, *Lactococcus lactis* 11/19-B1 strain, *Enterococcus mundtii* EM1s strain, *Klebsiella pneumoniae* ATCC10031 strain, *Salmonella enterica* ATCC14028s strain, *Serratia marcescens* 2170 strain, *Pseudomonas aeruginosa* PAO1 strain, *Escherichia coli* JM109 strain, and *E. coli* O-157 Sakai strain did not differ between agar media prepared with TSB of lot A or lot B (Figure

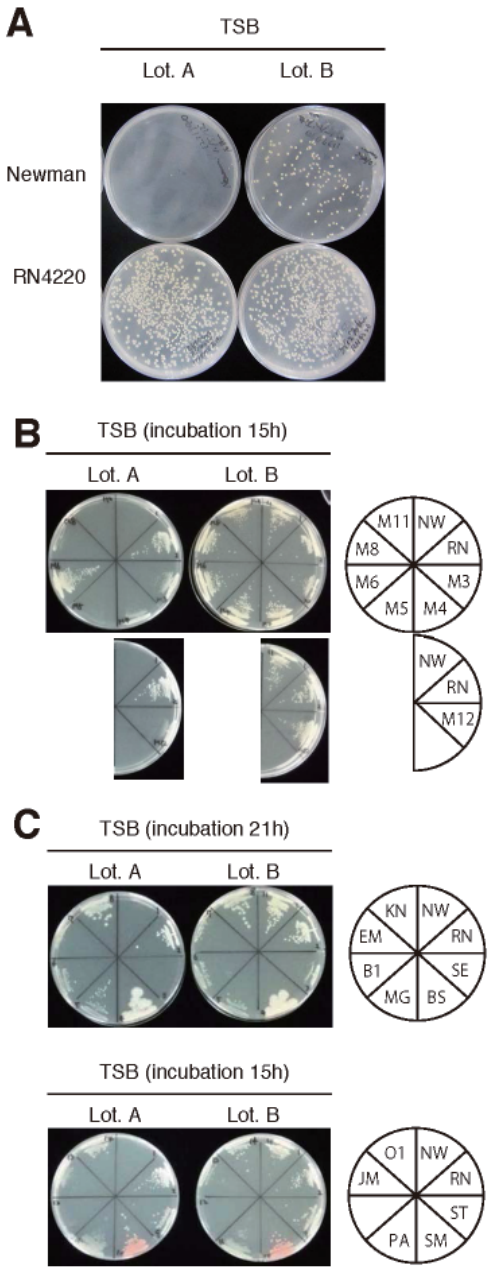

Figure 1. Cell growth inhibition of the Newman strain and MRSA strains on agar plates prepared with TSB of lot A. **(A)** Colony formation of the *S. aureus* Newman strain and the RN4220 strain on agar plate prepared with TSB of lot A or lot B was examined. The Newman strain and RN4220 strain were cultured overnight at 37°C in liquid medium prepared with TSB of lot B, the bacterial suspensions were diluted 1 × 10^6-fold, and 100-µL aliquots were spread on the agar plates, followed by incubation at 37°C for 24 h. **(B)** Growth of the *S. aureus* Newman strain, the RN4220 strain, and several MRSA strains. Fully grown bacterial cultures were diluted 1 × 10^2-fold with saline, and streaked by a loop on agar plates prepared with TSB of lot A and lot B followed by incubation at 37°C for 15 h. Strains are shown on the right side of the photographs. NW, Newman; RN, RN4220; M3, MRSA3; M4, MRSA4; M5, MRSA5; M6, MRSA6; M8, MRSA8; M11, MRSA11; M12, MRSA12. **(C)** Growth of various bacterial strains on agar plates prepared with TSB of lot A or lot B. Bacteria were cultured overnight at 37°C or at 30°C (MG1363 strain and 11/19-B1 strain), and streaked on agar plates by a loop. Plates were incubated at 37°C. Bacterial strains are shown on the right side of the photographs. NW, Newman; RN, RN4220; SE, ATCC12228; BS, 168; MG, MG1363; B1, 11/19-B1; EM, EM1s; KN, ATCC10031; ST, ATCC14028s; SM, 2170; PA, PAO1; JM, JM109; O1, O-157 Sakai.

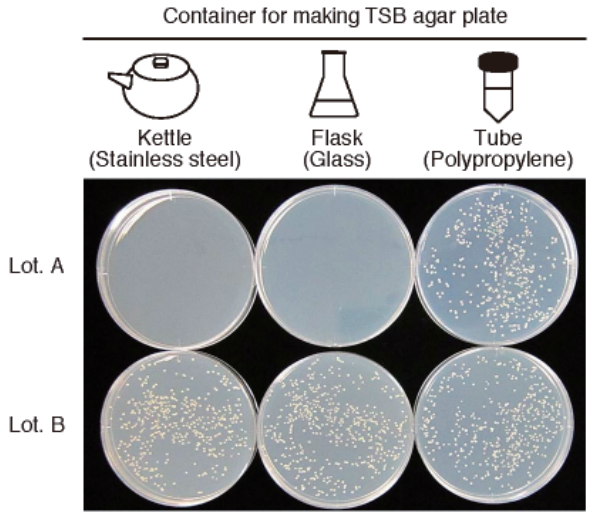

Figure 2. Effect of containers used to autoclave TSB-agar on cell growth of the Newman strain. The Newman strain was cultured at 37°C overnight in a liquid medium prepared with TSB of lot B. The culture was diluted 1 × 10^6-fold, spread on agar plates prepared with TSB of lot A or lot B autoclaved in different containers, and incubated at 37°C for 18 h.

1C). These findings suggest that TSB of lot A contained compounds that specifically inhibit the cell growth of certain strains of *S. aureus* and *S. epidermidis*.

3.4. *Effect of different containers used for autoclaving agar on the growth inhibition of Newman strain by TSB lot A*

Growth inhibition of the Newman strain by TSB of lot A was observed when the agar was autoclaved in a stainless steel container (kettle) or a glass container (conical flask), but not when it was autoclaved in polypropylene tubes (Figure 2). We considered the possibility that substances that eluted from the surface of the polypropylene tubes inactivated the compounds that inhibited the cell growth of the Newman strain. To test this, we autoclaved water for medium preparation in polypropylene tubes, and then prepared agar plates with TSB of lot A using water from the glass container. We examined whether the Newman strain would grow on the agar plates. The Newman strain did not form colonies on the agar plates (Figure 3B, plate 2), thereby excluding the possibility that substances that eluted from polypropylene during the autoclaving inhibited the cell growth inhibiting activity of TSB of lot A.

We then examined the possibility that the compound responsible for cell growth inhibition in TSB of lot A was absorbed on the surface of the polypropylene tubes during autoclaving. To test this, we compared the cell growth of Newman strain on agar plates prepared with TSB of lot A with or without pieces of polypropylene tubes in a glass container. The Newman strain did not form colonies on the agar plates prepared by autoclaving without the addition of pieces of polypropylene tubes (Figure 3B, plate 3). In contrast,

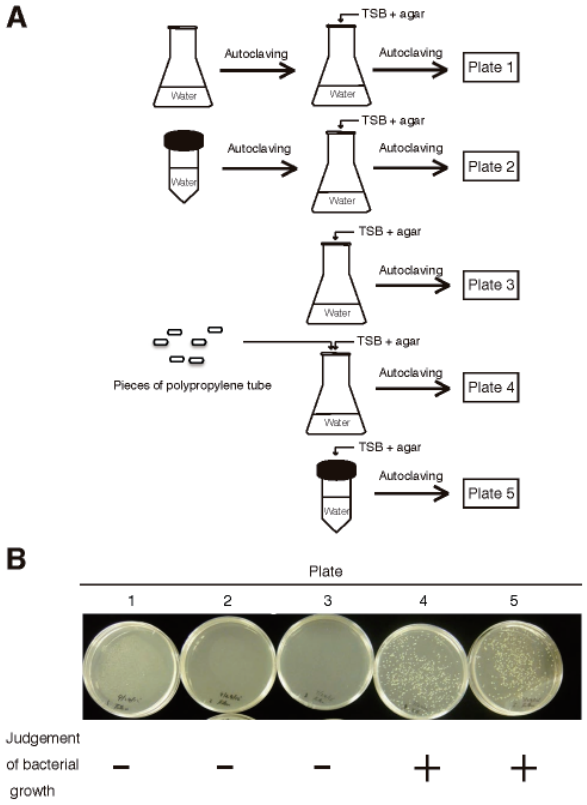

Figure 3. Effects of different preparations of TSB (lot A)-agar on growth of the Newman strain. TSB (lot A)-agar was autoclaved in glass containers (plates 1-4) or in a polypropylene tube (plate 5). **(A)** Schematic protocols for the preparation of TSB (lot A)–agar are shown. **(B)** Plate 1, Water was autoclaved in a glass flask, followed by repeat autoclaving with TSB (lot A) and agar in a glass flask. Plate 2, Water was autoclaved in a polypropylene tube, followed by autoclaving in a glass flask. Plate 3, TSB (lot A) and agar in water were autoclaved in a glass flask (control). Plate 4, TSB (lot A) and agar in water were autoclaved in a glass flask with pieces of polypropylene. Plate 5, TSB (lot A) and agar in water were autoclaved in a polypropylene tube. After gelation of the agar medium in Petri dishes, overnight cultures of the Newman strain were diluted 1×10^6-fold, and 100-µL aliquots were spread on agar plates, followed by incubation at 37°C for 18 h.

the Newman strain formed colonies on agar plates prepared by autoclaving with the addition of pieces of polypropylene tubes (Figure 3B, plate 4). These findings suggest that the compound responsible for cell growth inhibition of the Newman strain in TSB of lot A binds the surface of polypropylene tubes during autoclaving.

3.5. *Isolation of the polypropylene-binding compounds from TSB of lot A that inhibit cell growth of the Newman strain*

Cell growth inhibition of the Newman strain by TSB of lot A was observed not only on agar plates but also in a liquid medium in glass test tubes (Figure 4, left). The cell growth inhibitory effect against the Newman strain in the liquid medium with TSB of lot A was not observed, however, in polypropylene tubes (Figure 4, right). This finding suggests that the cell growth

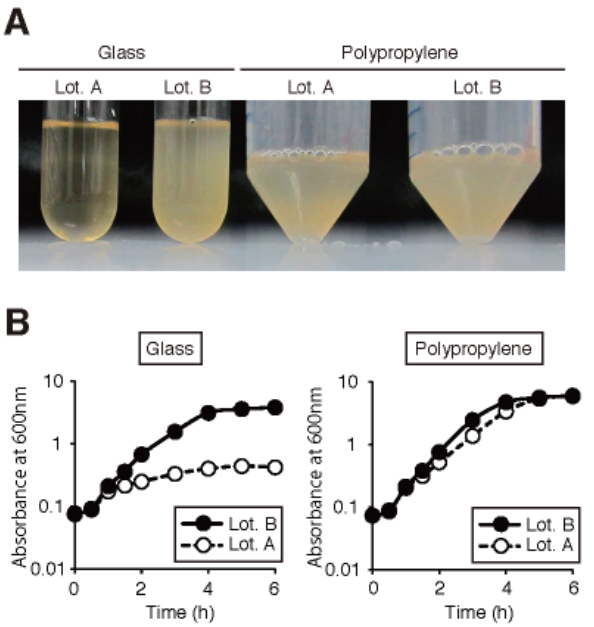

Figure 4. Effect of culture tubes on growth of the Newman strain in a liquid medium prepared with TSB of lot A. The TSB liquid medium (5 mL) from lot A or lot B was added to glass containers or polypropylene tubes. Aliquots (50 µL) of overnight culture of the *S. aureus* Newman strain were added and incubated at 37°C. Turbidity (absorbance at 600 nm) was measured. **(A)** Culture solutions after 3.5 h. **(B)** Left, culture in a glass container; Right, culture in a polypropylene tube. ○ , a TSB liquid medium of lot A. ●, a TSB liquid medium of lot B.

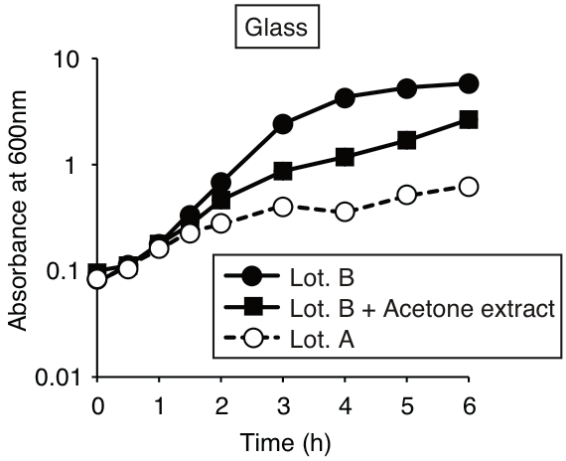

Figure 5. Inhibitory effect of polypropylene-binding substances from TSB of lot A on cell growth of the Newman strain. TSB medium from lot A was incubated overnight at room temperature in a polypropylene tube, followed by extraction with acetone. The acetone extract was added to the liquid medium of TSB (lot B). Fully grown cultures of the Newman strain were added, and incubated at 37°C. Aliquots were sampled and turbidity (absorbance at 600 nm) was measured. ○ , TSB of lot A; ■, TSB of lot B with the acetone extract; ●, TSB of lot B.

inhibitor present in TSB of lot A binds polypropylene not only under autoclave conditions, but also under a cultivation temperature of 37°C.

We attempted to isolate the polypropylene-binding compounds inhibiting the cell growth of the Newman

strain from TSB of lot A. The liquid medium of TSB of lot A prepared by autoclaving in a glass bottle was transferred to a polypropylene tube, and allowed to stand at room temperature. Then, the TSB liquid medium was decanted, and material adsorbed on the surface of the tube was extracted with acetone. Acetone was removed by evaporation, and a liquid medium prepared with TSB of lot B was added, followed by incubation of the Newman strain. Consequently, the medium containing the acetone extract fraction inhibited the cell growth of the Newman strain (Figure 5). This finding suggests that the compounds that inhibit the cell growth of the Newman strain are present in TSB of lot A, and that this compound binds to polypropylene.

4. Discussion

In the present study, we demonstrated that TSB of lot A contained compounds that inhibit the cell growth of the *S. aureus* Newman strain and a majority of MRSA strains. TSB contains tryptone and a soy extract (*3*). Compounds in TSB of lot A that inhibit the cell growth of the Newman strain probably originated from one of these natural products. Therefore, the amount of the cell growth inhibitory compounds in TSB probably varies greatly between different lots of TSB. Proper management of product lots in laboratories is crucial, especially given the importance of *S. aureus* as a clinical pathogen.

Although compounds in TSB of lot A severely inhibited cell growth of the Newman strain and a majority of MRSA strains, they did not inhibit cell growth of the RN4220 strain or several other MRSA strains. Therefore, the cell growth inhibiting effect of the compounds is specific to particular strains of *S. aureus*. Because the whole genome sequences of Newman and RN4220 have been determined (*4,5*) by comparative genomic analysis and recombinant technology of genes by phage transduction, genetic approaches will be applicable for dissecting the mechanism of selective cell growth inhibition against the Newman strain. The capacity of cell growth inhibiting compounds to absorb to polypropylene resins may allow us to purify and determine the structure of the compounds. Additional genetic and biochemical studies are necessary to further elucidate the compounds responsible for the inhibition of cell growth.

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Original Article

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Identification and methods for prevention of *Enterococcus mundtii* infection in silkworm larvae, *Bombyx mori*, reared on artificial diet

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Summary **Previously, it was reported that *Enterococcus mundtii* (*E. mundtii*) was associated with flacherie disease of silkworm larvae reared on artificial diet. In this study, we report that *E. mundtii* was isolated from diseased silkworm larvae, and validated as a pathogenic bacterium of the animal. When silkworm larva was infected with 1.04×10^6 colony-forming units of *E. mundtii* via oral administration of diet, half population died within six days, indicating that the bacterium is pathogenic to silkworm. Less severe infection was found to cause anorexia and hamper the development of larvae. This pathogen was found to proliferate in both time- and dose-dependent manner in the gastrointestinal tract of the animal. The bacterium was isolated from powder of artificial diet made from mulberry leaves, and from mulberry leaves growing at a field. Minimum inhibitory concentration determination revealed that this bacterium was susceptible to tested antibiotics. Vancomycin treatment of diet significantly decreased the number of *E. mundtii* in intestine of silkworm larvae infected with the bacteria, compared to control. Furthermore, autoclaving or gamma ray irradiation of diet was also effective for exclusion of *E. mundtii* from the diet without the loss of its nutrient capacities. These results suggest that mulberry leaves used in making artificial diet for silkworm larvae is one of the sources of *E. mundtii* infection; and that antibiotic treatment, autoclaving or gamma ray irradiation of artificial diet can exclude the bacteria.**

Keywords: Drug candidates, bacterial proliferation, gamma ray irradiation, pathogenic infection.

1. Introduction

Recent advances in pharmacy and medicine have brought to fore the need to develop disease models with invertebrate animals for evaluation of therapeutic behavior of drug candidates and novel pharmaceuticals. We are proposing use of silkworm as a model animal for this purpose. Apart from low cost of rearing, low possibility of bio-hazard problems, and easy injection with syringes during experimental researches; silkworm larvae as a non-mammalian animal also eliminate the ethical problems involved in studying mammals in preclinical tests for drug candidates (*1-4*). We recently discovered new antibiotic, lysocin E, from the cultured supernatant of *lysobacter sp*, a soil bacterium, by monitoring the therapeutic effects in silkworm model infected with *Staphylococcus aureus* (*5*).

Artificial diet-based mass production of silkworm larvae is a critical step for use of silkworm models for drug screening. However, infectious disease from pathogens is usually a major challenge sometimes leading to under-development, poor productivity and death of larvae. There have been previous reports of infection of silkworm larvae by various pathogens *vis-à-vis*: *Metarhizium anisopliae* (*6*), *Alphabaculovirus* (*7*), *parasitic Nosema species* (*8*), and *Bombyx* densovirus type 1 (*9*). Similarly, *Enterococcus mundtii* (*E. mundtii*) has been shown to be directly associated with flacherie disease of the silkworm larvae reared on artificial diet (*10*). In this present study, we explored the origin of observed pathogenic infection in industrial

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and transgenic strains of silkworm larvae, used as a non-mammalian animal model in drug discovery, and possibility of preventing such infections. We identified *E. mundtii* as the infectious pathogen and found that it came from artificial diets made from mulberry leaves. To confirm, we successfully isolated the bacteria from the leaves. Further results suggested that antibiotic treatment; autoclaving and gamma ray irradiation of artificial diet could handle the infection problems.

2. Materials and Methods

2.1. *Microorganisms and chemicals*

All used microorganisms were obtained from laboratory stock. Chloramphenicol and tetracycline were purchased from Nacalai Tesque Incorporation (Chuo-ku, Tokyo, Japan); while ampicillin, vancomycin, Sodium Hydroxide; and kanamycin were purchased from Wako Pure Chemical Industries (Mie-gun, Mie, Japan); and Tokyo Chemical Industries Ltd (Chuo-ku, Tokyo, Japan), respectively. Brain Heart Infusion (BHI) and Mueller Hinton Broths were purchased from Decton, Dickinson and Company (Franklin Lakes, New Jersey, USA).

2.2. *Determination of Minimum inhibitory concentrations of antibiotics*

For Minimum inhibitory concentrations (MICs) determination, bacteria were visually examined in two fold serial dilutions of tested samples in Mueller Hinton Broth medium according to National committee for clinical laboratory standards (*11*). Cultures were incubated at 37°C for 24 hours. MICs were determined using broth dilution method as described previously (*12*). The MIC was defined as the lowest concentration of the assayed antimicrobial agent (drug) that inhibited the visible growth of the *E. mundtii*, compared with that of the drug-free control, using the 96-well micro-titer plate format.

2.3. *Silkworm larvae, artificial diet, diet powder and mulberry leaves*

Fertilized eggs of silkworm, *Bombyx mori* (Hu.Yo x Tukuba.Ne) were obtained from Ehime Sanshu, Ehime, Japan. Artificial diet (Silkmate 2S) and diet powder (Silkmate 2M) purchased from Nosan Corporation (Yokohama, Kanagawa, Japan) were used for the experiment. Eggs were surface sterilized following the established procedure for germ-free rearing of larvae (*13,14*). Hatched larvae were raised by feeding on Silkmate 2S at room temperature till fifth instar before used for experiments. Larvae were sacrificed for gut analysis between 72 h and 120 h from the beginning of the 5th instar. Before sacrificing larvae, a disinfection treatment on the surface of larval body was carried out: larvae were dipped in 70% ethanol for 10 seconds. Mulberry leaves were harvested from the garden of the Graduate School of Agricultural and Life Sciences, The University of Tokyo (Bunkyo-ku, Tokyo, Japan).

2.4. *Identification of bacteria*

To identify all bacteria strains, single colonies were Gram stained (*15*). Bacterial species were determined by sequencing and analysis of 16S Ribosomal Ribonucleic Acid (16S rRNA) as previously described (*16*), with modification as follows: The genomic DNA was extracted from the 50 mL liquid Brain Heart Infusion (BHI) broth medium according to previous protocols (*17,18*). The 16S rRNA fragment was amplified using selected universal primers (both forward and reverse) in a GeneAmp Polymerase Chain Reaction (PCR) System 9700 thermal cycler with the following programs: 30 sec at 94°C, 30 sec at 55°C, and 1.5 min at 72°C for 35 cycles. The PCR reactions were terminated at 72°C for 7 min and thereafter, cooled at 4°C. To ascertain the specificity of the PCR amplification; negative control (PCR mix without DNA template) and positive control (chromosomal DNA of *E. mundtii*) were included. Amplification was confirmed by electrophoretic analysis of 5 µL PCR reaction mixtures on a 1% agarose gel, and was screened by ChampGel gel image disposal system. The PCR product was purified using a QIAamp Column DNA Gel Extraction Kit purchased from Qiagen (Chuo-ku, Tokyo, Japan) according to the manufacturer's instruction. The 16S rRNA was sequenced, and then analyzed with GenBank by Blast software.

2.5. *Examination of the effects of autoclaving, gamma ray irradiation or infection of diet on growth of silkworm larvae*

To understudy the effects of diet infection on growth, larvae were fed with either infected (2 g mixed with 10 µL of *E. mundtii* full growth culture) or non-infected artificial diet at 27°C for 24 h. The feeding with non-infected diet continued daily till the 6th day, weight of silkworm was measured and photo shots taken. To probe into the effects of severe heat treatment of diet powder and gamma ray irradiation on growth of larvae; thirty larvae were randomly divided into three groups: A, B and C (n = 10). Groups A and B, were fed with autoclaved (Silkmate 2M heat-treated to 121°C for 20 min using High Pressure-Steam Standard Autoclaving machine) and gamma ray irradiated diet (both freshly obtained from the manufacturer). While group C was fed with normal diet obtained from the laboratory stock (but also supplied by the same manufacturer). Feeding with same kind of diet in same proportion continued for 5 days. Total weights of larvae in different groups were measured daily.

2.6. *Determination of median lethal dose (LD_{50}) of E. mundtii in silkworm by oral administration*

LD_{50} of *E. mundtii* via oral administration was determined as follows: 50 µL of various dilutions of *E. mundtii* full growth culture were adsorbed into 5 g diets and fed to 5 silkworm larvae. The number of colony-forming units (CFUs) of *E. mundtii* administered per larva at which 50% of the tested population was dead after 6 days of administration was defined as LD_{50}.

2.7. *Isolation of E. mundtii from mulberry leaves*

Mulberry leaves were crushed with a sterilized teflon homogenizer in sterilized physiological saline. The aliquot was streaked on BHI agar plates, and incubated at 30°C for 24 h.

2.8. *Data and statistical analysis*

Data were analyzed using Graph-pad prism student *t*-test. Descriptive data were expressed as mean ± Standard error of mean (SEM). $p < 0.0001$ was considered statistically significant.

3. Results

3.1. *Identification of E. mundtii from feces of weakened silkworm reared on artificial diet*

We occasionally experienced disease problems in our non-mammalian animal model, both industrial strain (Hu.Yo x Tukuba.Ne) and strains of transgenic silkworms larvae, reared on artificial diet. The larvae looked weakened and showed symptoms of diarrhea. From previous reports that Silkworm larvae infected with various pathogens, including bacteria, suffer from severe diseases (*6-9*), we hypothesized that the problems might be due to infection. First, we tested whether the weakened silkworms were infected with bacteria. Feces obtained from the weakened silkworm were suspended in sterilized physiological saline and spread on BHI agar plates. Homologous colonies formed on the BHI agar plate (Figure 1A). These isolated colonies were round-shape and appeared purple-coloured when viewed through a microscope after Gram staining (Figure 1B), suggesting that the suspected pathogen is Gram-positive cocci. Sequencing analysis of its 16S rRNA gene revealed that the sequenced gene of the isolated pathogen matched with that of a bacterium specie, *E. mundtii*. All together, these results suggest that the weakened silkworms were infected with *E. mundtii*.

3.2. *Median lethal dose (LD_{50}) determination via oral administration*

We next examined whether or not the isolated *E. mundtii* was pathogenic to silkworm. Results indicated that when full growth was injected into hemolymph of silkworms, the animals were killed within 72 h. The killing effects by *E. mundtii* was also observed when bacteria were administered by oral pathway through feeding with diet adsorbed with the bacterial full growth, though it took longer period unlike direct injection into hemolymph. We determined the median lethal dose, LD_{50} and found that the number of the bacteria needed to kill half population of tested silkworm larvae via oral administration was 1.04×10^6 CFUs after 6 days (Figure 2). Taken together, the above results demonstrated that *E. mundtii* was pathogenic to silkworm larvae, *Bombyx mori*.

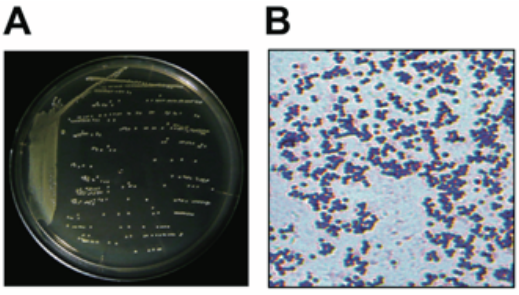

Figure 1. Isolation of colonies from feces of silkworm suffering from diarrhea. (A) Feces were obtained form silkworm with diarrhea, suspended in sterilized physiological saline and spread on BHI agar plates. The BHI plate was incubated at 30°C for 24 h. **(B)** Isolated single colony was suspended in sterilized physiological saline, Gram stained according to previous protocol (*15*) and observed with a microscope.

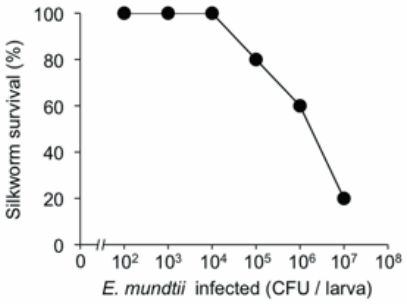

Figure 2. Lethal effects on silkworm by oral administration of *E. mundtii*. Various CFUs of *E. mundtii* were adsorbed onto artificial diet through the full growth culture. The infected diet was fed to larvae and incubated at 27°C. After 6 days, number of surviving silkworms ($n = 5$) was counted.

3.3. *Effects of mild E. mundtii infection on growth of silkworm larvae*

Next, we examined problems associated with infection of larvae with a smaller number of *E. mundtii*; much less than LD_{50}. Silkworm larvae were fed with artificial diet containing 1×10^5 CFUs/larva. The feeding was further continued for 5 days with non-infected artificial diet. Photo shot of the body sizes of silkworms was taken on the 6th day (Figure 3A). The weight of infected

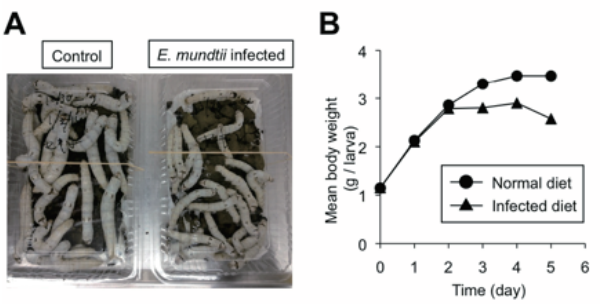

Figure 3. Inhibition of silkworm growth by oral administration of *E. mundtii*. (A) Silkworms were reared for 6 days at 27°C with normal diet (control) or diet containing 1×10^5 CFUs/larva of *E. mundtii* (*E. mundtii* infected). **(B)** Silkworms were reared at 27°C with normal diet or diet containing 1×10^5 CFUs/larva of *E. mundtii* (Infected diet). Body weight of the silkworms was monitored for 5 days and mean weight determined.

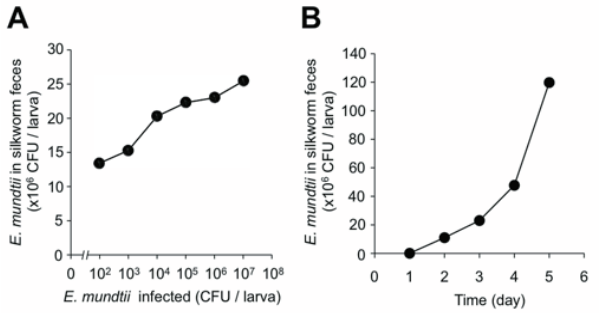

Figure 4. Proliferation of E. mundtii in silkworm larvae. (A) Various CFUs of *E. mundtii* (1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, and 1×10^7 CFUs/larva) were administered to silkworm via oral administration of diet infected with the full growth culture and bacterial population was monitored on day 3. **(B)** Larvae were infected (1×10^6 CFUs/larva) through infected diet administration and bacteria population monitored for 5 days. Five pieces of feces were obtained from each group (n = 5), suspended in sterilized physiological saline and spread on BHI agar plates. After incubation at 30°C for 24 h, the number of colonies that appeared on the plates was counted. Total *E. mundtii* CFUs present in feces were calculated.

larvae decreased in a time-dependent manner compared to non-infected (larvae) control, becoming very drastic beginning from 48 h post-infection (Figure 3B). Loss of appetite was also observed in the diseased animals. These results suggest that less severe *E. mundtii* infection of larvae results to anorexia and hampers the growth of the animals.

3.4. *E. mundtii proliferation in the gut of silkworm larvae*

Having observed the time-dependent detrimental effects of the infection, we next asked whether *E. mundtii* proliferated in the gut of silkworm larvae after oral ingestion. Silkworms were fed with artificial diet containing *E. mundtii*, and number of the bacteria in feces was determined by counting number of colonies after spreading of samples on agar plates followed by incubation. Results demonstrated that the bacterial population in the feces of larvae increased in both dose- and time-dependent manner (Figures 4A and 4B). These findings suggest that the bacteria proliferated in the gut (gastrointestinal tract) of silkworms, and that this could probably be responsible for its pathogenic effects in the host.

3.5. *Isolation of bacteria from artificial diet of silkworm*

Cappellozza *et al*. reported previously the infection of silkworm larvae reared on artificial diet by *E. mundtii* (*10*). Coupled with our observation, therefore, we suspected that the origin of *E. mundtii* was artificial diet. First, we attempted to isolate *E. mundtii* by incubation of pieces of the artificial diet in BHI medium. However, we could not obtain reproducible results of bacterial proliferation. Thus, we decided to examine the presence of *E. mundtii* on the powder of artificial diet before heat treatment. The manufacturer that provided the artificial diet (Silkmate 2S), which was heat-treated to cause agar polymerization, also provided the powder (Silkmate 2M) prior to heat treatment. Incubation of BHI agar plate that was streaked with suspension of the powder lead to appearance of bacteria colonies within 24 h. Sequencing and analysis of isolated bacterial 16S rRNA gene indicated that *E. mundtii* was present as a major population. Other colonies were identified by sequence of 16S rRNA as *Acinetobacter baumanni*, *Stenotrophonomonas matophilia*, *Pseudomonas geniculata*; and *Enterococcus gallinarum*, *casseliflavus*, and *faecalis* sub-specie. These findings suggest that the powder of the artificial diet prior to heat treatment contains viable *E. mundtii* cells and other bacteria.

3.6. *E. mundtii isolation from mulberry leaves*

Since the artificial diet contained powder made from mulberry leaves, we hypothesized that the origin of the infection was mulberry leaves. To test this, we examined the presence of the bacteria on the leaves. To establish a method for isolation of *E. mundtii* from mulberry leaves phylosphere, which may contain multiple microbial florae other than *E. mundtii*, we attempted to find growth conditions specific for *E. mundtii*. Since we have demonstrated that *E. mundtii* proliferated in intestine of silkworm under alkaline conditions, and corroborated by previous reports (*19,20*), we hypothesized that *E. mundtii* was resistant to alkaline conditions. To test this, we compared the growth of *E. mundtii* and *Staphylococcus aureus* or *Pseudomonas aeruginosa* (common environmental bacteria) on agar BHI plates with various concentrations of Sodium Hydroxide (NaOH). We found that among other specie of bacteria only *E. mundtii* could form colonies on plates containing 0.06 N NaOH, whereas the growth of *S. areus* and *P. aeruginosa* were terminated under the condition (Figure 5A). This information allowed

us to establish a selection method for *E. mundtii* from other bacteria. By using alkaline agar plates containing 0.06 N NaOH, we readily isolated bacterial colonies from mulberry leaves, which could grow under alkaline conditions (Figure 5B). The sequence of the 16S rRNA gene indicated that majority of bacteria on the alkaline plates were *E. mundtii*. From 1.0 g mulberry leaf sample, we isolated 2.6×10^5 bacteria colonies out of which 1×10^4 colonies were *E. mundtii*. These results suggest that the mulberry phyloplane contains *E. mundtii* among other epiphytes.

3.7. *Vancomycin treatment of diet decreased E. mundtii population in silkworm gut*

From above results, we hypothesized that small number of *E. mundtii* cells remains viable even after mild heat-treatment of artificial diet (employed by the manufacturer), and this could be responsible for the observed infection. Hence, we decided to establish a method to exclude the bacteria from silkworms. First, we attempted to exclude *E. mundtii* by treatment with antibiotics. To choose an appropriate antibiotic, we determined the MICs of ampicillin, vacomycin, kanamycin, chloramphenicol and tetracycline against *E. mundtii*. Among five antibiotics tested, vancomycin showed most potent activity for inhibiting the growth of *E. mundtii* as summarized in Table 1. These results suggest that the bacterium is highly susceptible to vancomycin treatment. Thus, we next examined whether the addition of vancomycin to diet would cause the disappearance of *E. mundtii* from intestine of silkworms infected with the bacteria. We added full growth of the bacteria to artificial diet followed by larvae feeding with the diet containing vancomycin (160 µg/g diet). Then, we examined the presence of the bacteria in the intestinal materials of the larvae. The results indicated that feeding of vancomycin-treated diet caused significant decrease ($p < 0.0001$) in the number of isolatable *E. mundtii* colonies, from average of 5.7×10^6 CFUs in the intestine of control to 3.6×10^3 CFUs in the treated animals 24 h post-administration of diet (Figure 6). From these results, we propose that vancomycin treatment could prevent *E. mundtii* infection in silkworm larvae possibly by inhibiting in the proliferation process *in-vivo*.

3.8. *Autoclaving and gamma ray irradiation of the artificial diet exclude E. mundtii*

High pressure-heat-based processing, and irradiation are two established methods of food sterilization that

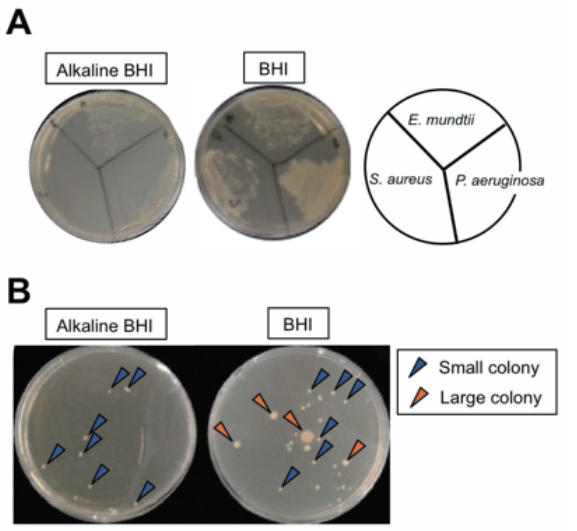

Figure 5. Isolation of *E. mundtii* from mulberry leaves using alkaline containing agar plate. (A) Single colonies of *E. mundtii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* were streaked on both BHI agar plate with 0.06 N NaOH (Alkaline BHI plate) and BHI agar plate (BHI). The plates were incubated at 30°C for 24 h. **(B)** Mulberry leaves were homogenized in sterilized physiological saline. The homogenized suspension was streaked on both BHI agar plate with 0.06 N NaOH (Alkaline BHI plate) and BHI agar plate (BHI). The plates were incubated at 30°C for 24 h.

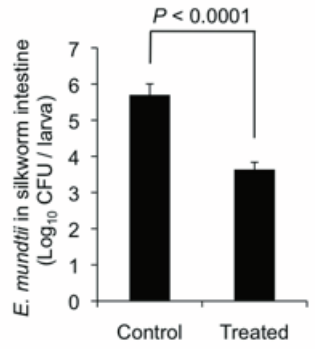

Figure 6. Vancomycin treatment inhibits proliferation of *E. mundtii* in silkworm. Larvae were fed with *E. mundtii* infected diet (overnight culture of E. mundtii, 5 µL/g diet) treated with vancomycin (160 µg/g diet) or without (control) for 24 h. Intestinal materials of silkworms were harvested and the number of CFUs determined. All data were analyzed using Graph-pad prism student *t*-test. Descriptive data ($n = 5$) were expressed as mean ± SEM. Statistical probability, $p < 0.0001$ was considered significant.

Table 1. MIC values of common antibiotics against *E. mundtii*

Strain	Minimum Inhibitory Concentration (µg/mL) of antibiotic				
	Ampicillin	Vancomycin	Chloramphenicol	Kanamycin	Tetracycline
E. mundtii	8	0.5	32	32	1

The minimum inhibitory concentration (µg/mL) was determined using broth dilution method of antimicrobial susceptibility testing as described previously (*11,12*).

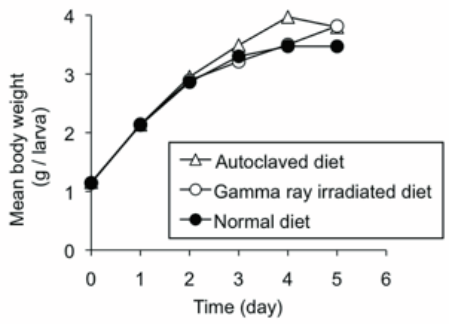

Figure 7. Autoclaving and gamma ray treatment of diet do not affect silkworm growth. Autoclaved diet (saline containing diet powder heat-treated to 121°C for 20 min) was prepared from Silkmate 2M. Gamma ray irradiated diet was prepared from artificial diet, Silkmate 2S. Silkworms (n = 10) were fed with normal diet, autoclaved diet or gamma ray irradiated diet at 27°C for 5 days. Body weight of the silkworms was monitored for 5 days and mean weight determined.

inactivate pathogenic bacteria employed in the food industry (*21,22*). Thus, we tested for the effects of these two methods; autoclaving and gamma ray treatment on the presence of the bacteria on the diet on one hand, and on the growth of fed animals on the other. When water suspension of diet powder (Silkmate 2M) was autoclaved at 121°C for 20 min, no bacteria colony was observed by examination of the materials spread on nutrient agar plates (data not shown), confirming our hypothesis that severe heat treatment of diet materials could kill the bacteria. The measurement of the weight of silkworms fed with the diet revealed that the autoclaving of the artificial diet did not affect the growth of the larvae (Figure 7). Gamma ray irradiation of the artificial diet also gave the result of complete loss of living bacteria. Silkworm larvae fed with the gamma ray irradiated diet also showed normal growth (Figure 7), compared to the normal diet fed animals. These findings suggest that autoclaving or gamma ray treatment of larvae diet powder or diet during preparation has no negative consequences on growth of the fed animals.

4. Discussion

In this current study, we observed the presence of *E. mundii* in intestine of diseased silkworm larvae, *Bombyx mori*, reared with artificial diet, which posed a serious challenge in screening of drug candidates. Our present results indicate that feeding of diet containing *E. mundtii* caused proliferation of the bacteria in silkworm gut. Infection of larvae by the bacteria led to weight loss, loss of appetite, and eventually caused death of larvae. Therefore, we concluded that *E. mundii* was pathogenic to silkworm larvae, *Bombyx mori*, hence poses a direct threat to experimental reproducibility during research; and should be excluded. We traced the origin of the bacteria and successfully isolated it from diet powder made from mulberry leaves. We also demonstrated that mulberry leaves contained E. mundii. Therefore, we concluded that the mulberry leaves was a source of infection. We tested the sensitivity of antibiotics against *E. mundtii*, and demonstrated that the bacteria showed high susceptibility to vancomycin treatment *in vitro*. *In vivo*, we found that when silkworm larvae were fed with vancomycin-treated infected diet (160 μg/g diet), the bacterial proliferation was significantly decreased (< 1%) in the intestine. Thus, we propose that vancomycin treatment is a possible method to exclude *E. mundii* infection from silkworm, although the complete loss of the bacteria was very difficult. Furthermore, we established that autoclaving or gamma ray irradiation of diet completely removed *E. mundtii* without affecting its nutrient content. However, when larvae were fed with mulberry leaves, *E. mundtii* proliferated in the gut although no apparent problem of growth of larvae was observed. We hypothesize that substances(s) present in mulberry leaves might regulate the pathogenicity of the bacteria in the body of the silkworm host. Another postulation is the possibility that co-existence of other bacteria in intestine contribute to avoid infection problems posed by *E. mundtii*. Further investigation is, therefore, needed to understand the mechanism by which silkworms escape from the *E. mundtii* infection problem even when fed with mulberry leaves infested with the pathogen.

In conclusion, this study identified the origin of *E. mundtii*, validated the bacteria as a pathogen of silkworm larvae and proposed methods for preventing the infection.

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Original Article

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Differentially expressed proteins in fluconazole-susceptible and fluconazole-resistant isolates of *Candida glabrata*

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Summary **The current study aimed to identify the differences presented in the proteome of fluconazole-susceptible isolates of *Candida glabrata* compared to those with fluconazole-resistant ones. Two-dimensional differential gel electrophoresis was applied to identify proteins that were differentially expressed in fluconazole-susceptible and fluconazole-resistant isolates of *C. glabrata*. Eight proteins including aspartyl-tRNA synthetase, translation elongation factor 3, 3-phosphoglycerate kinase, ribosomal protein L5, coproporphyrinogen III oxidase, pyruvate kinase, G-beta like protein, and F1F0-ATPase alpha subunit were found to be more abundantly represented, while four proteins including vitaminB12-(cobalamin)-independent isozyme of methionine synthase, microtubule-associated protein, adenylosuccinate synthetase, and aldose reductase were found to be less abundantly represented in fluconazole-resistant strains versus those with fluconazole-susceptible ones. These differentially expressed proteins were primarily associated with energy metabolism, stress response, and macromolecule synthesis. Proteins associated with energy metabolism, stress response, and macromolecule synthesis may play a role in the development of fluconazole resistance in the clinical isolates of *C. glabrata*. Multiple different mechanisms are involved in the development of fluconazole resistance in *C. glabrata*. These findings provide a scientific basis for discovering new genes and mechanisms associated with fluconazole resistance in *C. glabrata*.**

Keywords: *Candida glabrata*, proteomics, fluconazole, drug resistance

1. Introduction

In recent years, mucous membrane and invasive infections caused by *Candida glabrata* have increased significantly. In some areas, *C. glabrata* has become one of the three most frequent causes of candidiasis besides C. albicans (*1*). Currently, triazole antifungals are the most commonly used agents to treat *Candida* infections, however, *C. glabrata* exhibits intrinsically low susceptibilities to triazoles (*2*). The drug resistance of *C. glabrata* has become a major problem affecting the efficacy of clinical treatment of *C. glabrata* infections.

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Recently, studies have been conducted to understand the mechanisms of fluconazole resistance in *C. glabrata*. It is relatively clear that the *ERG11*, *CDR1* and *CDR2* genes are associated with the development of fluconazole resistance in *C. glabrata* (*3,4*), but these genes can only partially explain the resistance in clinical isolates of *C. glabrata*. So far there have been no other genes reported to be responsible for the development of drug resistance. Therefore, it would be helpful to explain the mechanisms of fluconazole resistance in *C. glabrata* if more resistance-related genes and mechanisms could be identified. Over the years, genomic technologies have been the primary ways to study the resistance mechanisms of fungi. Recently, proteomics, defined as the global analysis of cellular proteins, is becoming a key area of research that is developing in the post-genome era. It has been demonstrated to be a powerful tool for the investigation of complex biochemical processes and the discovery

of new proteins. Proteomics provides a new tool for the study of fungal resistance. The evaluation of protein profiles in response to a modification of fungal resistance could represent a valid and useful approach for the development of new therapeutic strategies. Discovering resistance-related proteins through proteomics makes it possible to identify additional genes and mechanisms related to the development of fungal resistance. At present, proteomics are being applied to study the pathogenesis and drug resistance mechanisms of fungi.

In the present study, proteomic techniques were applied to identify proteins that are differentially expressed in fluconazole-resistant isolates of *C. glabrata* and fluconazole-susceptible ones. The study aimed to find new resistance-related proteins and thus provide a point of reference for the discovery and study of new fluconazole resistance related genes and mechanisms.

2. Materials and Methods

2.1. *Strains and antifungal susceptibility testing*

Clinical strains of *C. glabrata* were isolated from clinical specimens. The *in vitro* susceptibilities of *C. glabrata* isolates to fluconazole were determined by the broth microdilution method as described in the NCCLS document M27-A2 (*5*). All susceptibility tests were carried out in duplicate. The interpretive criteria for susceptibility to fluconazole were based on those published by the NCCLS (*5*) and were as follows: susceptible, ≤ 8 μg/mL; susceptible-dose-dependent (S-DD), 16 to 32 μg/mL; and resistant, ≥ 64 μg/mL. Nineteen isolates of *C. glabrata* were included in the present study, 10 of them were fluconazole-susceptible isolates while 9 of them were fluconazole-resistant isolates.

2.2. *Protein extraction*

Isolates of *C. glabrata* were cultured on Sabouraud dextrose agar containing chloramphenicol for 24 h. After that, each isolate was diluted in YPD broth (1% yeast extract, 2% peptone, 1% dextrose) and grown overnight at 30°C in a shaking incubator. Yeasts were grown in YPD at 30°C until they reached the late exponential phase of growth (optical density at 600 nm, approximately 7). Fungal cells were harvested, and washed with phosphate buffered saline (PBS) for 3 times. The 9 resistant isolates were then mixed with the same amount of cells for the isolation of proteins, while the 10 susceptible ones were also mixed with the same amount of cells to isolate proteins, respectively. The detailed extraction process was as follows: added an equal volume of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) lysis buffer [8 mol/L urea, 2 mol/L thiourea, 65 mmol/L dithiothreitol (DTT), 0.5% pharmalytes pH 3-10, 0.5 mmol/L phenylmethanesulfonyl fluoride, 4% 3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate, 1% nonidet P-40, 0.05% nuclease mix] into the mixture of isolates, re-suspended the precipitation, then transferred into a mortar. Poured appropriate amount of liquid nitrogen and the sample immediately formed white lumps. Afterwards, ground the lumps into powder with continuously adding liquid nitrogen during the grinding process. After 10 min of grinding, the slurry was transferred into an EP tube. The residual liquid in the mortar was rinsed with a small amount of 2D-PAGE lysis buffer and the solution was transferred into the EP tube as well. Extracted on ice for half an hour by shaking the tube every several minutes. Centrifuge at 12,000 revolutions per minute (RPM) for 15 min. The resulting supernatant contained the soluble protein fraction. Soluble sodium dodecylsulphate (SDS) lysis buffer (100 mM Tris-HCl (pH = 6.8), 20% glycerol, 4% SDS, 100 mM DTT) was added into the insoluble precipitate after the extraction with 2D-PAGE lysis buffer. Ice extraction was conducted for 30 min by shaking every several minutes. Centrifuge at 12,000 RPM for 15 min. The resulting supernatant contained the less soluble protein fraction. Concentration of soluble protein was analyzed using a micro-Bradford assay using a Protein Assay Kit II (Bio-Rad, Hercules, CA, USA). Concentration of less soluble protein was then determined by Bio-Rad's DC-RC kit (Bio-Rad, Hercules, CA, USA).

2.3. *2D-PAGE and imaging*

2D-PAGE was conducted according to the methods described in the references (*6,7*). After electrophoresis, the gel was ripped off, silver nitrate or Coomassie blue-stained, and imaged with the Image Scanner.

2.4. *SDS-PAGE separation*

In order to identify less soluble proteins, the insoluble precipitate after extraction with 2D-PAGE lysis buffer was re-extracted with SDS-containing lysis buffer (100 mM Tris-HCl (pH = 6.8), 20% glycerol, 4% SDS, 100 mM DTT). After determination of the protein concentration with the DC-RC method, the precipitate was split into 100 μg/tube and boiled in water for 5 min. It was then loaded into the comb holes for SDS-PAGE. Discontinuous SDS-PAGE electrophoresis vertical plate with gel concentration of 11.5% and stacking gel concentration of 4.8% was used for SDS-PAGE.

2.5. *Image master software analysis*

After repeated 2D-PAGE, the gel was scanned with an Image Scanner, and three pairs of parallel samples were

prepared for analysis with GE Image Master software (version 5.0) in order to identify protein spots with differences greater than 2-fold.

2.6. *In-gel digestion*

Protein digestion was conducted according to the method described by Hellmann *et al*. (*8*) with minor modifications.

2.7. *Liquid chromatography ion trap tandem mass spectrometry (LC-MS/MS)*

The mass spectrometer used was a Bruker ESI ion trap mass spectrometer equipped with a nano-liquid chromatography spray source. All measurements were carried out under positive ion mode. Five peptides with maximum intensity (beyond the threshold) were selected at each time for MS/MS analysis. The acquired data was entered into the database (NCBInr), and compared by the MS/MS Ions Search function in the Mascot software (Matrix Science Ltd., UK) (*http://www.matrixscience.com*). Peptide with Mowse scores higher than the threshold would be regarded as an identified peptide, and its corresponding proteins would be regarded as identified proteins. For proteins with only one matching peptide, its ion score should be more than 40, and inspected manually to check whether the map had good quality (whether the sample peak was apparent, and signal to noise ratio was acceptable) and whether there were four or more consecutive y ions or b ions (*e.g.*, y2, y3, y4, y5) as well as whether the protein molecular weight and isoelectric point were consistent with the position on the gel. The identification result should have the highest score and should be from the species of *C. glabrata*. Amino acid sequence of the identification result was entered into Génolevures database for Blast (*http://cbi.labri.fr/Genolevures/blast.php#*) to search for homologous proteins corresponding to *Saccharomyces cerevisiae*.

3. Results

3.1. *SDS-PAGE*

Figure 1 shows the insoluble proteins separated by SDS-PAGE. It could be concluded that the SDS-PAGE maps of fluconazole-susceptible and fluconazole-resistant isolates of *C. glabrata* are basically the same, with no differing bands.

3.2. *2D-PAGE separation*

Soluble proteins were separated by 2D-PAGE, with sample volume of 250 μg for silver staining and 500 μg for Coomassie blue staining, and the strips were pH 3-10L. A pair of representative gel diagrams is

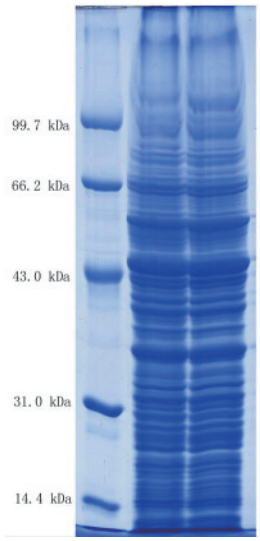

Figure 1. SDS-PAGE map of less soluble proteins of *C. glabrata* (left: Marker; middle: resistant isolates; right: susceptible isolates).

presented in Figure 2. Both of the up-regulated protein spots in the susceptible strains (marked in Figure 2A, 2D-PAGE map of susceptible isolates) and the up-regulated protein spots in the resistant strains (marked in Figure 2B, 2D-PAGE map of resistant isolates) were shown in Figure 2. It was demonstrated that protein spots were well separated, gel diagrams were consistent, and the different points were evident.

3.3. *Image master software analysis*

Any points of difference were subject to Image Master software analysis and rigorous screening. The gels of the two samples were grouped, and then matched within group to reduce human error in each gel production process. A minimum 2-fold change was considered for up-regulated proteins spots

3.4. *Mass spectrometry of differential protein spots*

All differential protein spots obtained were subject to gel digestion with trypsin, and the sequence of extracted peptides was analyzed by mass spectrometry. Twelve differential protein spots were accurately identified. All identification results are shown in Table 1. Among the 12 proteins, eight [aspartyl-tRNA synthetase, translation elongation factor 3, 3-phosphoglycerate kinase, ribosomal protein L5, coproporphyrinogen III oxidase, pyruvate kinase, G-beta like protein, and F1F0-ATPase alpha subunit] were found to be more abundantly represented, while four [vitaminB12-(cobalamin)-independent isozyme of methionine synthase, microtubule-associated protein, adenylosuccinate synthetase, and aldose reductase] were found to be less abundantly represented in fluconazole-resistant strains versus those with fluconazole-susceptible ones. These proteins are involved in energy metabolism, stress

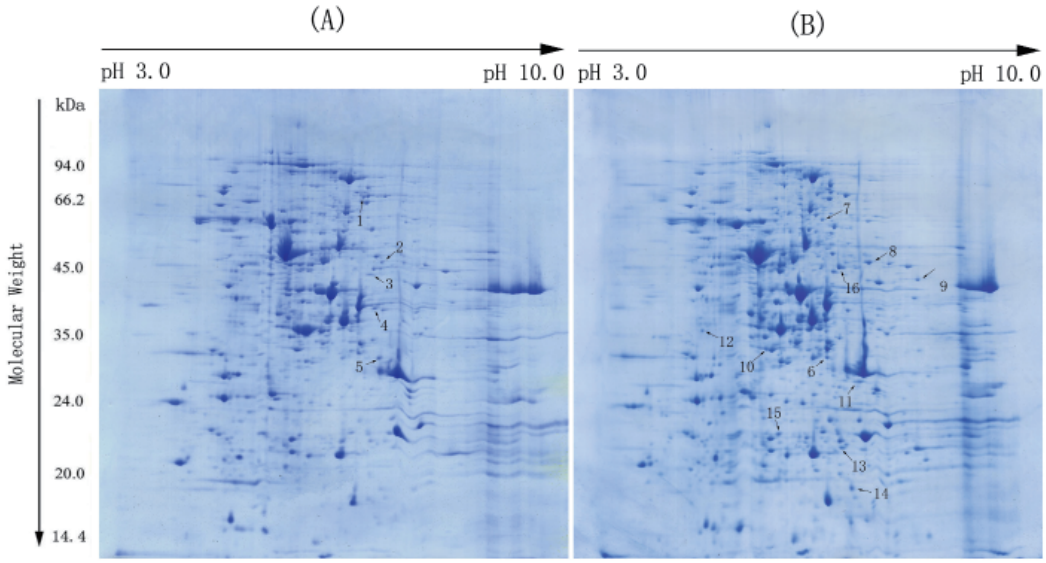

Figure 2. Soluble *C. glabrata* proteins resolved by 2D-PAGE for *C. glabrata* strains (A: susceptible isolates; B: resistant isolates). Spots representing differentially expressed proteins that were identified by mass spectrometry are correspondingly numbered in Table 1. Four proteins (highlighted) were found to be up-regulated in susceptible isolates, 12 protein spots (highlighted) up-regulated in resistant isolates.

Table 1. Differentially expressed proteins identified by mass spectrometry

Spot	Accession No.[a]	Protein (description)[b]	Score	pI	Mw (kDa)	Protein coverage
Up-regulated proteins which exhibited a 2-fold or greater change in susceptible isolates						
1	gi|50290071	YER091c (vitaminB12-(cobalamin)-independent isozyme of methionine synthase)	288	6.07	85809	11%
2	gi|50293659	YOR272w (microtubule-associated protein)	62	6.37	51047	2%
3	gi|50292025	YNL220w (adenylosuccinate synthetase)	789	6.35	48377	42%
4	gi|50289743	YHR104w (aldose reductase)	111	6.4	37460	14%
Up-regulated proteins which exhibited a 2-fold or greater change in resistant isolates						
5	gi|50286523	YLL018c (aspartyl-tRNA synthetase)	520	5.98	63322	28%
6	gi|50285389	YLR249w (translation elongation factor 3(EF-3))	155	5.71	116678	6%
7	gi|50293403	YCR012w (3-phosphoglycerate kinase)	422	6.37	44704	30%
8	gi|50294105	YPL131w (ribosomal protein L5 (L1a)(YL3))	344	6.42	33741	36%
9	gi|50292617	YDR044w (coproporphyrinogen III oxidase)	324	6.08	37720	20%
10	gi|50294908	YAL038w (pyruvate kinase)	301	6.08	54975	20%
11	gi|50286153	YMR116c (G-beta like protein)	213	5.85	30297	36%
12	gi|50294700	YBL099w (F1F0-ATPase alpha subunit)	563	8.99	58599	26%

[a] Accession numbers from NCBInr database; [b] Name and decription of *Saccharomyces cerevisiae* homologous proteins as denoted in the Génolevures database.

response, and biosynthesis of macromolecules in *C. glabrata*.

4. Discussion

In previous studies (*9,10*), sets of matched susceptible and resistant isolates, both derived from the same strain, were used to investigate the molecular mechanisms underlying the development of azole resistance in *Candida* species. These isolates, however, were rarely available in clinical settings. Therefore, those studies were limited either in that only a relatively small number of clinical isolates were investigated or the isolates originated from a single patient (*9*). Resistant isolates used in some studies were formed by laboratory induction (*10*). However, it is currently not clear whether the mechanisms of resistance identified in resistant isolates formed by laboratory induction are the same as those resistant in arbitrarily selected isolates. In this study, genetically unmatched clinical *C. glabrata* isolates from different patients was used to investigate proteomic differences between the susceptible and resistant isolates of *C. glabrata*.

Proteomic technologies were applied in this study to identify the proteomic expression differences between fluconazole-resistant and fluconazole-susceptible isolates of *C. glabrata*. Twelve differentially expressed proteins were identified, 8 of them were up-regulated in the resistant isolates while 4 were up-regulated in the susceptible isolates. These differential proteins may be implicated in the resistance of *C. glabrata* to fluconazole, however their relationship with

fluconazole resistance needs to be confirmed by further studies. These proteins are mainly involved in energy metabolism, stress response, and biosynthesis of macromolecules in *C. glabrata*. Rogers *et al*. (*10*) conducted proteomic analysis of experimentally induced fluconazole resistance in *C. glabrata* and identified a number of differentially expressed proteins involved in glycolysis and glycerol metabolism, oxidative stress and biosynthesis of macromolecules. The authors also reported two differentially expressed proteins, 3-phosphoglycerate kinase and fecal porphyrin original III oxidase, while 10 differentially expressed proteins identified in our study have never been reported so far. These differential proteins reflect the physiological, biochemical, metabolic and other changes with the development of resistance of *C. glabrata* to fluconazole, but their specific roles in the development of resistance still need to be further studied.

This study failed to identify any differential expression of proteins that have been confirmed to be associated with fluconazole resistance, such as Cdr1p (encoded by *CDR1* gene), Cdr2p (encoded by *CDR2* gene), Erg11p (lanosterol 14α-demethylase, encoded by *ERG11* gene). The lack of changes in the membrane proteins in our study contradicts published results on protein changes as well as our previous results on gene expression proving significant increase in the expression of *ERG11*, *CDR1* and *CDR2* genes linked to azole resistance in *C. glabrata* clinical isolates (*11*). This inconsistency may originate from the protein isolation and preparation. These three proteins are membrane proteins. The former two are ATP-binding transporter proteins, while the last one is the target enzyme for fluconazole. Fungal membrane proteins are highly hydrophobic and fungi have cell walls, so isolation of such proteins is very difficult. In order to improve protein isolation efficiency, grinding in liquid nitrogen was applied in this study to fully release the proteins in cell walls. Meanwhile, the study also adopted step-wise protein extraction methods by referring to methods in the literature which extracted soluble proteins and insoluble proteins respectively, and separated them with 2D-PAGE and SDS-PAGE, respectively, to improve the separation efficiency of insoluble proteins. However, the study ultimately failed to detect the differential expression of insoluble proteins, while mainly soluble proteins were detected. Therefore, the extraction scheme of fungal proteins and two-dimensional gel electrophoresis remain to be further improved and optimized. In addition, the treatment with liquid nitrogen during the extraction procedure and the use of mortar should have had effect on the cell wall.

Progress has been made in some studies by examining drug resistance mechanisms of *C. glabrata* through protein levels. Niimi *et al*. (*12*) found that after exposure to fluconazole, *C. glabrata* can quickly induce the expression of two membrane proteins Cdr1p and 14-DM of *Candida*. These two membrane proteins changed from barely detectable levels to become the main composition of the cell membrane. To further confirm that CDR1 overexpression is associated with drug-resistance, the authors studied the functional expression of the *CDR1* gene in Saccharomyces cerevisiae and found that after the *CDR1* gene was transferred into Saccharomyces cerevisiae that lacked other protein-coding genes, the isolates changed from fluconazole-susceptible to highly fluconazole-resistant. Additionally, the expression of the membrane proteins Cdr1p were also found to be up-regulated. Marichal *et al*. (*9*) found that through a comparative analysis of protein 2D-PAGE maps of fluconazole-resistant *Candida* isolates and susceptible isolates that at least 25 proteins had increased expression, while 76 proteins had decreased expression. It was believed that these differentially expressed proteins may be related to drug-resistance related proteins, however, differential proteins were not identified. As a result, it was not identified which specific proteins may be related to drug-resistance. Rogers *et al*. (*10*) carried out a proteomic study on laboratory-induced fluconazole-resistant isolates of *C. glabrata* and detected a total of 25 differential proteins, in which expressions of Cdr1p and 14-DM in drug-resistant isolates were increased. It was believed that in addition to up-regulated expressions of Cdr1p and 14-DM related to drug-resistance of *C. glabrata*, other differential proteins might also be a cause of the formation of drug-resistance. These studies examined drug-resistance mechanisms of *C. glabrata* from protein levels, and investigated other possible mechanisms of the formation of drug-resistance, but such studies (including ours) did not carry out further studies on the specific functions of differential proteins. Therefore, the specific role of these differential proteins in the formation of drug-resistance of *C. glabrata* remains to be further studied and confirmed.

Proteomics has provided a powerful tool for the study of resistance mechanisms of *C. glabrata* and has enabled its in-depth study. However, proteomics research of *C. glabrata* is still in its initial stages and there are still many issues to be further addressed. One major issue so far is that only a limited number of open reading frames have been identified in *C. glabrata* genome, restricting the identification of the *C. glabrata* proteins. Additionally, techniques for extraction, separation and identification of *C. glabrata* proteins are to be further improved and optimized. Many *C. glabrata* resistance-associated proteins are membrane proteins. Due to their strong hydrophobicity and poor solubility, only a very limited number of membrane proteins can be obtained through the current protein preparation methods and these proteins cannot be effectively extracted. Also, some

of the functional proteins have low expression and cannot be displayed with the current color-developing methods (*13*) as well as some low-abundance proteins that can be shown in 2D-PAGE gel cannot be fully identified by mass spectrometry. With the development and advances of proteomics technology, proteomics research will play a greater role in the studies of drug-resistance mechanisms of *C. glabrata*.

Some limitations to our study should be noted. We found 12 kinds of differential proteins in fluconazole-susceptible and fluconazole-resistant isolates of *C. glabrata*, but there were no gene level data about these proteins or comparable function in these pathogens. We have not done the real-time PCR to confirm protein level data. We used pooled isolates for the extraction of the proteins for both susceptible and resistant strains. The clinical strains were not studied individually in terms of proteomics. Such a performance may have flattened the results and we were not able to know whether or not there are specific differences in the single strains. Therefore, the differential proteins identified in this report should be interpreted with caution.

In conclusion, this study used proteomics technologies to isolate and identify 12 proteins that may be associated with the development of resistance of *C. glabrata* to fluconazole, including energy metabolism-related enzymes, stress response proteins and macromolecular synthesis-related proteins. This study has demonstrated from protein levels that energy metabolism-related enzymes, stress response proteins and macromolecular synthesis-related proteins are all likely involved in the formation of drug-resistance of *C. glabrata* and further confirmed that the formation of drug-resistance of *C. glabrata* is the result of multiple mechanisms. We have also provided a theoretical basis for the discovery and study of new drug-resistance related genes and drug-resistance mechanisms.

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Original Article

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Hispidin and related herbal compounds from *Alpinia zerumbet* inhibit both PAK1-dependent melanogenesis in melanocytes and reactive oxygen species (ROS) production in adipocytes

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Summary **Recently several compounds from Okinawa plants including *Alpinia zerumbet* (alpinia) were shown to inhibit directly the oncogenic/ageing kinase PAK1 (p21-activated kinase 1). Furthermore, it was recently revealed that both PAK1 and PAK4 (p21-activated kinase 4) are equally essential for the melanogenesis in melanoma cells. Thus, in this study, we tested if several alpinia compounds inhibit the melanogenesis in melanoma (B16F10) cells, as well as the PAK1-dependent up-regulation of both reactive oxygen species (ROS) and nitric oxide (NO) in cultured adipocytes (3T3-L1) without any cytotoxicity. The effect of alpinia compounds on the melanogenesis was measured by both the melanin content and intracellular tyrosinase activity in melanoma cells treated with 3-isobutyl-1-methylxanthine (IBMX), a melanogenesis stimulating hormone. We found that (1E,3E,5E)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran (MTD), 5,6-dehydrokawain (DK), labdadiene, hispidin and dihydro-5,6-dehydrokawain (DDK) at 50 μg/mL reduced the melanin content by 63-79%. The MTD, DK and hispidin, at 50 μg/mL, inhibited tyrosinase activity by 70-83% in melanoma cells. Among these compounds, labdadiene, MTD, (E)-2,2,3,3-Tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1H-quinolizine (TMOQ) and hispidin strongly inhibited the ROS production. Hispidin, labdadiene and MTD at 20 μg/mL inhibited NO production by over 70%. These findings altogether suggest that some of these alpinia compounds could be potentially useful for the prevention or treatment of hyper-pigmentation and obesity.**

Keywords: *Alpinia zerumbet*, ROS, melanogenesis, MTD, labdadiene, hispidin, PAK1

1. Introduction

Alpinia zerumbet (alpinia) belongs to the Zingiberaceae, which widely distributes in South-East Asia areas such as Taiwan and Okinawa Islands. As a traditional folk medicine, it has been used for the treatment of flu and pain (*1*). Several years ago, extract of alpinia seeds was found to have a potent hypo-lipidemic effect *in vivo* (*2*). More recently, a variety of pharmacological activities have been reported on isolated alpinia compounds. For instance, both 5,6-dehydrokawain (DK) and dihydro-5,6-dehydrokawain (DDK) inhibit the major oncogenic kinase PAK1 (RAC/CDC42-activated kinase 1) (*3*) and block the platelets aggregation which depends on PAK1 (*4*). Furthermore, DK and DDK also inhibit PAK1-dependent viral infection by interfering with both HIV-integrase and neuraminidase (*5,6*). Beside, labdadiene has an anti-atherosclerosis activity and inhibits lipid peroxidation, cyclo-oxygenases and cancer growth (*7,8*). Hispidin, a metabolite of DK, also inhibits a few oncogenic kinases including protein kinase C (PKC)

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and PAK1, and possesses the anti-obesity and anti-cancer activities (*9,10*).

Melanin pigments, eumelanin and pheomelanin, are responsible for dark skin color (*11*), and protect the skin from UV-induced injury (*12*). However, the over-production of melanin in the skin leads to pigmentary disorders including dryness of the skin, irregular pigmentation-freckles, lentigines, wrinkling, and inelasticity (*13*). Melanin is synthesized in the melanocytes *via* an enzymatic cascade with tyrosinase as a key enzyme, and expression of tyrosinase gene depends on a few oncogenic/melanogenic transcription factors such as beta-catenin and microphthalmia-associated transcription factor (MITF), that are inactivated by a variety of herbal polyphenols such as tiliroside from raspberry and CAPE (caffeic acid phenethyl ester) from propolis (*14,15*). Interestingly, beta-catenin is among the direct substrates of the oncogenic kinases such as PAK1 and PAK4 (CDC42-activated kinase 4).

Very recently it was revealed that the melanogenesis in skin cells depends on two distinct members of PAK family, the oncogenic kinases PAK1 and PAK4 (*16*, Be Tu *et al*., manuscript submitted). Each appears to contribute independently by around 50%. Furthermore, we recently demonstrated that a variety of compounds from Okinawa plants, including hispidin, DK and DDK from alpinia, directly inhibit PAK1 (*3*). Moreover, we recently found that alpinia extract significantly extends the healthy lifespan of *Caenorhabditis elegans* (*17*). The phenotype of this worm treated with alpinia extract is very similar to that of PAK1-deficient mutant (RB689) of this worm which lives longer than the wild-type by more than 50% (*18*).

Thus, in this study, we tested if several compounds from alpinia rhizome or seeds such as hispidin, DK, DDK, labdadiene,(1E,3E,5E)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5-dihydrofuran (MTD) and (E)-2,2,3,3-Tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1H-quinolizine (TMOQ) inhibit the melanogenesis as well as both reactive oxygen species (ROS) and nitric oxide (NO) production in cultured 3T3-L1 adipocytes which are associated with obesity and known to be PAK1-dependent, as a PAK1-blocker called CAPE inhibits the melanogenesis as well as obesity and ROS/NO production (*15,19-21*). The outcome of our present study suggests that some of these alpinia compounds would be useful for treatment of both PAK1/PAK4-dependent hyper-pigmentation and PAK1-dependent obesity.

2. Materials and Methods

2.1. *Plant materials and reagents*

The rhizomes and seeds of alpinia were collected from Ryukyus University campus in Okinawa, Japan.

Insulin, Oil Red O and nitro blue tetrazolium (NBT) tablets were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). 3T3-L1 cells and B16F10 melanoma cells were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). Calf serum (CS) was obtained from Thermo Scientific (Old Highway, Tauranga, New Zealand). Dulbecco's modified Eagle medium (DMEM), dexamethasone, 3-isobutyl-1-methyl xanthine (IBMX), sodium nitrite, fetal bovine serum (FBS), triton-X, and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All reagents were of the highest grade available.

2.2. *Preparation of DK, DDK and hispidin from alpinia*

DK and DDK were isolated from the alpinia rhizomes as described previously (*6*) (Figures 1a and 1b).The conversion of DK to hispidin by rat liver microsomal protein was performed according to Tang *et al*. (*22*) with a few minor modifications (Figure 2).

2.3. *Preparation of labdadiene, MTD and TMOQ from alpinia*

Labdadiene was isolated from the alpinia seeds. The seeds (500 g) were extracted with 1 L of ethanol by maceration at room temperature for 2 days. After filtration, the filtrate was evaporated to dryness to give 3.26 g of dark syrupy extract. The extract was suspended in distilled water (300 mL) and partitioned with hexane (300 mL) and ethyl acetate (EtOAc) (300 mL).The EtOAc extract (1.02 g) was subjected to glass chromatography column containing silica gel (Silica gel 60N, particle size 63-120 μm, 70-230 mesh ASTM) and eluted with hexane: acetone (0-100%) to afford three fractions, fraction 1 was further used thin layer chromatography (TLC). The solvent used for isolate labdadiene (hexane: acetone, 9: 1, v/v). The TLC pre-coated silicagel (Merk-60 254, 0.25 mm thick) plate were developed using a Camag twin-through glass tank which was pre-saturated with the mobile phase for 45 min and each plate was developed to a height about 10 cm. After development plate was removed and dried and sports were visualized in UV light. Then, preparative thin layer chromatography (PTLC) was used to collect labdadiene, 50 mg from fraction 1 were dissolved in acetone and the plate was developed in hexane: acetone (9:1, v/v) with total volumes of 200 mL. Four bands were marked (under 254 nm) and then scraped from the plate. The scraping from each band was extracted (2-3 times), by stirring with EtOAc. Band 1 (major broad band) when extracted with EtOAc, gave 21.2 mg of steroid. Band 2 gave 5.6 mg, band 3 gave 6.0 mg and band 4 gave 18.2 mg. ^{1}H-NMR spectra were obtained in methanol-d_4 with a ULTRASHIELD^{TM} PLUS 500 MHz (Bruker Biospin, Germany). Chemical shifts (δ) were obtained

using standard pulse sequences on TopSpin 2.1 program Version 2.1.0 and reported in part per million (ppm). The signals are expressed as singlet (*s*), doublet (*d*), triplet (*t*), quarter (*q*) and multiplet (*m*). Coupling constants (*J*) are reported in Hz. Finally, band 3 is labdadiene with *m/z* (rel. int.); 302 (20), 137 (100), 123 (50), 109 (35), 95 (73), 81 (70), 69 (55), 55 (48), 41 (50). ^{1}H ($CDCl_3$): δ 0.74, 0.84 and 0.90 (*s*, each 3H, CH_3, 18, 19, 20), 1.04-2.51 (*m*, 14H, CH_2, *CH*, 1, 2, 3, 5, 6, 7, 9, 10, 11), 3.45 (*s*, 2H, CH_2, 14), 4.39 (*s*, 1H, CH_2, 17), 4.88 (*s*, 1H, CH_2, 17), 6.78 (*t*, 1H, *CH*, 12), 9.42 (*s*, 1H, *CHO*, 15) and 9.67 (*s*, 1H, *CHO*, 16) (Figure 1c).

The MTD was isolated by our laboratory. Briefly, air-dried rhizomes of alpinia (1000 g) were extracted with ethanol (1.5 L) for 2 days at room temperature. After evaporation of the solvent extract was obtained this dissolved in distilled water (300 mL) and defatted with hexane (300 mL). The defatted aqueous extract was further fractionated with chloroform ($CHCl_3$) (200 mL) and then EtOAc (200 mL). The EtOAc fraction was subjected to glass chromatography column containing silica gel (Silica gel 60N, particle size 63-120 µm, 70-230 mesh ASTM) and eluted with petroleum ether: $CHCl_3$ (0-100%) to afford three fractions. 2,5-bis (1*E*,3*E*,5*E*)-6-methoxyhexa-1,3,5-trien-1-yl)-2,5- dihydrofuran, HREIMS *m/z* 285.1 [M^+] (calcd for $C_{18}H_{22}O_3$, 286.16). IR *v* (KBr) cm^{-1}: 669, 1,646, 2,341, 2,359. ^{1}H-NMR (500 MHz, MeOD-d_4): 3.83 (*q*, 3H, OCH_3, J = 5.5 Hz, 9), 5.62 (*d*, 1H, *OCH*, 2), 6.24 (*d*, 1H, *CH*, 7), 6.86 (*d*, 1H, *CH*, 6), 7.58 (*s*, 1H, *CH*, 5), 7.59 (*d*, 1H, *CH*, 4), 7.14 (*q*, 1H, *CH*, J = 5.0 Hz, 1), 7.41 (*t*, 1H, *CH*, J = 7.0 Hz, 3), 7.36 (*t*, 1H, *CH*, J = 4.0 Hz, 8). ^{13}C-NMR (500 MHz, MeOD-d_4): 57.01 (C-9), 89.42 (C-2), 102.78 (C-7), 120.05 (C-6), 128.63 (C-5), 129.14 (C-4), 129.99 (C-3), 130.55 (C-1), 136.66 (C-8) (Figure 1d).

The TMOQ was also isolated by our laboratory. The seeds (100 g) of alpinia were extracted with 500 mL of methanol by maceration at room temperature for 2 days. After filtration, the filtrate was evaporated to dryness to give 21.6 g of dark syrupy extract. The extract was suspended in distilled water (500 mL) and partitioned with hexane (500 mL) and EtOAc (500 mL). The EtOAc extract (11.07 g) was subjected to glass chromatography column containing silica gel, eluted with methanol (MeOH) in dichloromethane (CH_2Cl_2) in a step gradient manner from 1% to 50% to obtain four fractions. Fraction 4 was further purified by the same column and condition as described above. (*E*)-2,2,3,3-tetramethyl-8-methylene-7-(oct-6-en-1-yl)octahydro-1*H*-quinolizine, HREIMS *m/z* 317.55 [M^+] (calcd for $C_{22}H_{39}N$, 317.2). IR *v* (KBr) cm^{-1}: 1,024, 1,121, 1,456, 1,507, 1,541, 1,558, 1,646, 1,698, 1,748, 2,360, 2,927, 3,448. ^{1}H-NMR (500 MHz, MeOD-d_4): 0.75 (*q*, 3H, CH_3, J = 10.5 Hz, 20), 0.85 (*t*, 3H, CH_3, J = 1.5 Hz, 18), 0.89 (*q*, 3H, CH_3, J = 4.0 Hz, 21), 0.94 (*t*, 3H, CH_3, J = 2.5 Hz, 1), 1.09 (*t*, 2H, CH_2, J = 2.5, 8), 1.17 (*q*, 2H, CH_2, 11), 1.22 (*q*, 2H, CH_2, J = 1.5 Hz, 14), 1.28 (*s*, 2H, CH_2, 14), 1.39 (*q*, 1H, *NCH*, J = 2.5 Hz, 12), 1.43 (*q*, 2H, CH_2, J = 2.2 Hz, 4), 1.58 (*t*, 3H, CH_3, J = 2.0 Hz, 19), 1.64 (*q*, 2H, CH_2, J = 4.5 Hz, 7), 1.75 (*q*, 2H, CH_2, J = 2.0 Hz, 5), 2.00 (*d*, 2H, NCH_2, 13), 2.26 (*d*, 2H, CH_2, 6), 2.47 (*t*, 2H, NCH_2, J = 5.5 Hz, 17), 3.27 (*t*, 2H, CH_2, J = 8.0 Hz, 22), 6.02 (*q*, 1H, *CH*, 3) and 6.36 (*d*, 1H, *CH*, 2). ^{13}C-NMR (500 MHz, MeOD-d_4): 14.00 (C- 21), 15.48 (C-20), 20.16 (C-4), 20.39 (C-19), 22.37 (C-18), 24.53 (C-5), 31.77 (C-7), 34.02 (C-15), 34.50 (C- 1), 37.79 (C-6), 39.90 (C-16), 40.36 (C-8), 42.03 (C-11), 43.39 (C-14), 55.96 (C-9), 63.34 (C-12), 65.83 (C-17), 68.81 (C-13), 108.85 (C-22), 122.20 (C-2), 135.00 (C- 3), 150.77 (C-10) (Figure 1e).

2.4. *Inhibition by alpinia compounds of melanogenesis*

2.4.1. *Cell culture*

Murine B16F10 melanoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000 U/100 µg/mL) at 37°C in a humidified atmosphere containing 5% CO_2.

2.4.2. *Cell viability*

Cell viability was determined using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, as described by Campos *et al*. (*23*). Briefly, B16F10 cells were plated at a density of 7 × 10^3 cells/well in a 96-well plate. After 48 h of culture, cells were exposed to varying concentrations of alpinia compounds (100 and 200 µg/mL) or 500 µM kojic acid and incubated for an additional 48 h at 37°C. Following incubation, the medium was removed, and the cells were washed twice with phosphate buffer and incubated with MTT solution (0.5 mg/mL) for 3 h at 37°C. The medium was discarded, and 200 µL of ethanol was added. The absorbance of each well was measured at 570 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.4.3. *Measurement of melanin content*

Melanin content was determined as described by Yoon *et al*. (*24*). In brief, B16F10 cells were plated at a density of 7 × 10^3 cells/well in a 96-well plate. After 48 h of culture, cells were exposed to varying concentrations of alpinia compounds (20 and 50 µg/mL) or 500 µM kojic acid. After 1 h, 100 µM isobutyl-1-methylxanthine (IBMX) was added and incubated for an additional 48 h at 37°C. The cells were washed twice with phosphate buffer, and then dissolved in 100 µL NaOH (1 N) containing 10% dimethyl sulfoxide (DMSO). Samples were incubated at 80°C for 1 h, and mixed to solubilize the melanin. The optical density of the mixed homogenate was measured at 490 nm. To measure the

amount of melanin in each experiment, the total amount of melanin (100%) produced during the experimental period was considered as the control group, and the rate of inhibition in the treatment groups was calculated in proportion to this standard.

2.4.4. *Intracellular tyrosinase activity*

Tyrosinase activity was determined as described by Li *et al*. (*25*) with slight modifications. B16F10 cells were plated at a density of 7×10^3 cells/well in a 96-well plate. After 48 h of culture, cells were exposed to varying concentrations of alpinia compounds (20 and 50 μg/mL) or 500 μM kojic acid. After 1 h, 100 μM IBMX was added and incubated for an additional 48 h at 37°C. The cells were then washed with ice cold phosphate buffer and lysed with phosphate buffer (pH 6.8) containing 1% Triton-X (90 μL/well). The plates were frozen at -80°C for 30 min. After thawing and mixing, 10 μL of 1% L-DOPA was added to each well. Following incubation at 37°C for 2 h, the absorbance was measured at 490 nm.

2.5. *Inhibition by alpinia compounds of ROS and NO production in cultured 3T3-L1 adipocytes*

2.5.1. *Cell culture and differentiation*

3T3-L1 cells were grown to confluency in Dulbecco's modified eagle's medium (DMEM) with 2% glutamine and 10% calf serum (CS) (v/v). Two days after reaching confluency, the cells were stimulated to differentiate into adipocytes by growing for an additional two days in DMEM that contained 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone, and 10 μg/mL insulin. Cells were then maintained in DMEM with 10% FBS and 10 μg/mL insulin for another two days, followed by culturing in DMEM with only 10% FBS for an additional four days. At that time, greater than 90% of the cells were differentiated 3T3-L1 adipocytes with accumulated lipid droplets. Differentiated 3T3-L1 cells were treated with different concentrations of the test compounds and maintained at 37°C in a humidified incubator containing an atmosphere of 5% CO_2 throughout the experiments.

2.5.2. *3T3-L1 cell viability assay*

Cell viability was measured using the MTT assay. The 3T3-L1 preadipocytes were seeded at a density of 1×10^4 cells per well in 96-well plates and incubated in culture medium. The cells were then treated with various concentrations of the tested compounds range from 100 and 250 μg/mL. After 72 h, the cells were incubated in the dark with an MTT solution for 4 h at 37°C. The supernatants were aspirated, DMSO was added to each well, and the plates were agitated to dissolve the formazan crystal product. Absorbance was then measured at 570 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Inc, Hercules, CA, USA). The percentage of viable cells was calculated by defining the cell viability without treatment as 100%.

2.5.3. *Measurement of intracellular ROS*

The 3T3-L1 cells were plated at a density of 2×10^6 cells/mL onto 96-well plates and cultured to confluency and differentiation as the method described above. ROS production was detected by nitro blue tetrazolium (NBT) assay (*26*). NBT is reduced by ROS to a dark-blue, insoluble form of NBT called formazan. After differentiation, cells were incubated the tested compounds at the various concentrations range from 10 and 20 μg/mL for 24 h. Cells then were incubated for 90 min with 100 μL in phosphate-buffered saline (PBS) containing 0.2% NBT. The dark-blue formazan was dissolved in 50% acetic acid, and its absorbance was determined at 570 nm.

2.5.4. *Measurement of NO production*

Cells were seeded and differentiated on 96-well plates as described previously. The nitrite formation (NO_2) assay used has been reported earlier by Fang *et al*. (*27*). After differentiation, cells were incubated with alpinia compounds, 10 and 20 μg/mL, for 24 h. Individual supernatants (100 μL) and Griess reagent (100 μL, 1:1 mixture (v/v) of 1% sulfanilamide and 0.1% napthyethylenediamine dihydrochloride in 5% H_3PO_4) were mixed in a separate 96 well plates and incubated for 10 min at room temperature. Absorbance was measured at 540 nm using a microplate spectrophotometer and nitrite concentrations were estimated using a standard curve generated for $NaNO_2$.

2.6. *Statistical analysis*

Data are expressed as mean values with their standard errors. Statistical comparisons were performed by oneway ANOVA followed by Duncan's multiple-range test. Statistical analysis was conducted using SAS (release 9.2; SAS Institute, Cary, NC, USA) and $p < 0.05$ was considered significant.

3. Results

3.1. *Anti-melanogenic effects of alpinia compounds*

Many cosmetic and pharmaceutical products have tried to find safe and effective inhibitors for melanogenesis. Especially, inhibitors for melanin formation and tyrosinase can be used for the inhibition of cellular pigmentation since melanin producing process involved a series of enzymatic and non-enzymatic oxidation (*11*). Thus, to examine the effect of alpinia compounds on

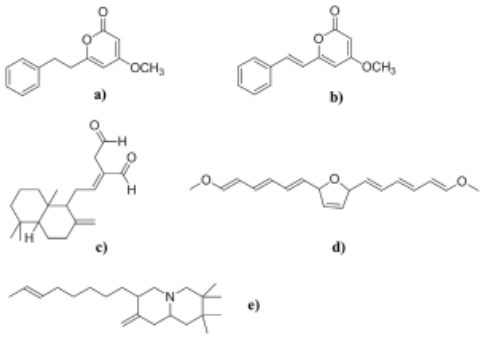

Figure 1. Chemical structure of DDK (1a), DK (1b), Labdadiene (1c), MTD (1d), TMOQ (1e).

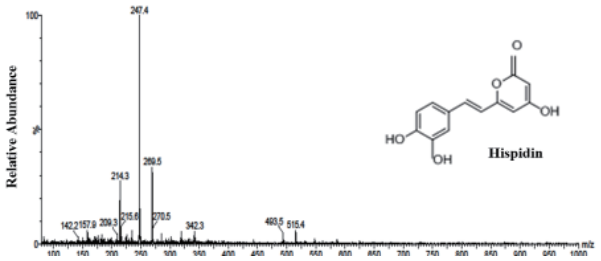

Figure 2. MS/MS spectra of hispidin (the peak 247.4) obtained by LC/MS analysis of the rat liver microsomes incubated with DK in the presence of NADPH-generating system.

melanogenesis, we measured both melanin content and intracellular tyrosinase activity.

First of all, we examined their effect on the viability *per se* of B16F10 melanoma cells by incubating them with each test compound for 48 h. As shown in Figure 3, none of these compounds (up to 100 μg/mL) significantly affected the cell viability.

3-Isobutyl-1-methylxanthine (IBMX) is an often used potent melanogenic stimulator (*28*), which up-regulates tyrosinase(s). Thus, in the presence of IBMX, we evaluated the anti-melanogenic activity of alpinia components.

3.1.1. *Melanin content*

The B16F10 melanoma cells were treated with each alpinia compound (20-50 μg/mL) in the presence of IBMX for 48 h. As shown in Figure 4, all treatments significantly ($p < 0.05$) reduced the melanin content. At 50 μg/mL MTD, DK, DDK, labdadiene, hispdin and TMOQ inhibited the melanin synthesis by 79%, 72%, 68%, 67%, 63% and 59%, respectively. Under same conditions, kojic acid (500 μM), a positive control, inhibited the melanin synthesis by 50.5%.

3.1.2. *Inhibition of intracellular tyrosinase activity*

To evaluate whether alpinia compounds inhibit intracellular tyrosinase activity or not, the B16F10 melanoma cells were treated the tested compounds (20-50 μg/mL) for 48 h, followed by incubation with L-DOPA. After treatment at 20 μg/mL, tyrosinase

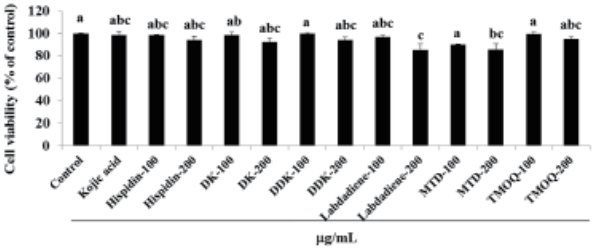

Figure 3. Effect of alpinia compounds on B16F10 melanoma cell viability. B16F10 cells were treated with tested compounds at either 100 or 200 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h. The percentage of viable cells was calculated by defining the cell viability without treatment as 100%. Reported values are the means ± SE ($n = 3$). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

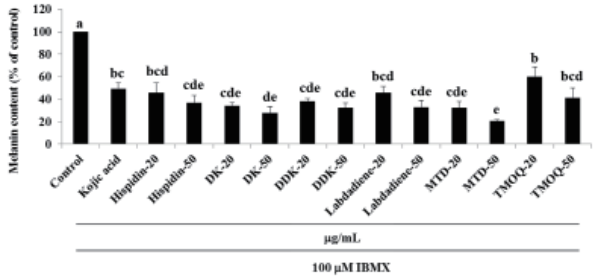

Figure 4. Effect of alpinia compounds on melanin production in B16F10 melanoma cells. B16F10 cells were treated with tested compounds at either 20 or 50 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h. Reported values are the means ± SE ($n = 3$). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

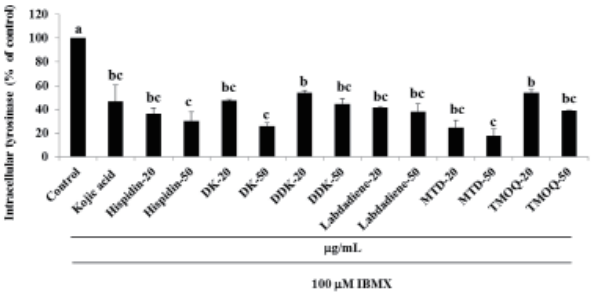

Figure 5. Effect of alpinia compounds on intracellular tyrosinase activity in B16F10 melanoma cells. B16F10 cells were treated with tested compounds at either 20 or 50 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h. Reported values are the means ± SE ($n = 3$). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

activity was inhibited by 76%, 64%, 59%, 52%, 46%, and 46% with MTD, hispidin, labdadiene, DK, TMOQ and DDK, respectively. At 50 μg/mL, all the tested compounds inhibited tyrosinase more potently than the positive control kojic acid (53%) (Figure 5). Among these compounds, MTD, DK and hispidin inhibited more strongly the tyrosinase activity by 83%, 74% and 70%, respectively, than labdadiene, TMOQ and DDK which inhibited the tyrosinase by 62%, 61% and 55%, respectively, without damaging the melanocyte viability.

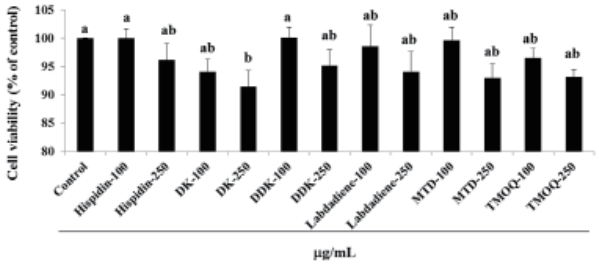

Figure 6. Effect of alpinia compounds on 3T3-L1 viability. 3T3-L1 cells were treated with tested compounds at either 100 or 250 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h. The percentage of viable cells was calculated by defining the cell viability without treatment as 100%. Reported values are the means ± SE (n = 3). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

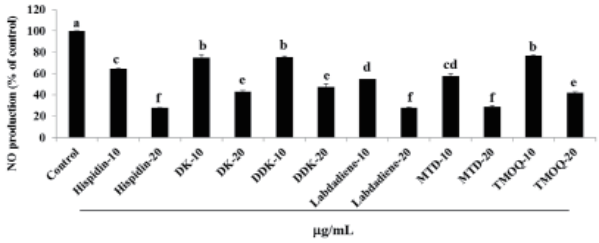

Figure 8. Effects of alpinia compounds on intracellular NO. 3T3-L1 adipocytes were treated with tested compounds at either 10 or 20 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Reported values are the means ± SE (n = 3). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

3.2. *Effects of alpinia on ROS and NO production in cultured 3T3-L1 adipocytes*

3.2.1. *Effect of alpinia compounds on 3T3-L1 cell viability*

To examine whether alpinia compounds have effect on cells, 3T3-L1 cells were incubated with them (100-250 μg/mL) for 72 h. At 100 μg/mL none of compounds showed any effect on the cell viability. At 250 μg/mL, DK, MTD, TMOQ, labdadiene, DDK and hispidin only slightly decreased cell viability by 9, 7, 7, 6, 5 and 4%, respectively (Figure 6).

3.2.2. *Measurement of intracellular ROS*

We found that alpinia compounds strongly inhibited the ROS production in cultured adipocytes. At 20 μg/mL ladadiene, MTD, TMOQ and hispidin in particular strongly inhibited the ROS production by 64%, 60%, 53% and 46%, respectively (Figure 7). DK and DDK showed to prevent ROS production with percentage inhibition values of 44% and 42%.

3.2.3. *Measurement of intracellular NO*

The NO production was also strongly inhibited by

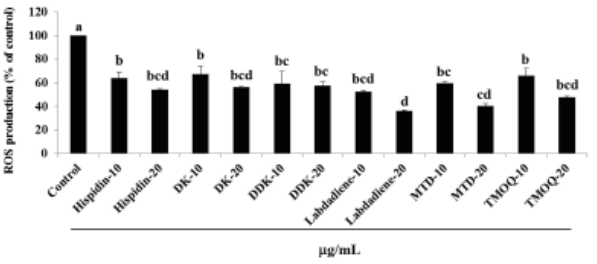

Figure 7. Effects of alpinia compounds on intracellular ROS production. 3T3-L1 adipocytes were treated with tested compounds at either 10 or 20 μg/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Reported values are the means ± SE (n = 3). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) than the control.

alpinia compounds (Figure 8). For example, labdadiene, hispidin and MTD at 20 μg/mL reduced the NO production by 72%, 72%, and 71%, respectively, while TMOQ, DDK and DK were less effective (by 58%, 57%, and 52%, respectively).

4. Discussion

We revealed here that several alpinia compounds are potent anti-melanogenic agents. At the concentration of 50 μg/mL, MTD, DK, hispidin and labdadiene in particular are effective anti-melanogenic agents, reducing both melanin content and intracellular tyrosinase by more than 50%. Furthermore, they are the stronger anti-melanogenic agents than kojic acid, which is a standard skin-lightening agent for treating melanogenesis and related diseases.

Furthermore, this study revealed that these alpinia compounds strongly inhibit both ROS and NO production in the cultured 3T3-L1 adipocytes, suggesting that alpinia is a good source for anti-oxidants, blocking both ROS and NO production.

In conclusion, the present study demonstrates that alpinia rhizome or seed is a safe and effective source for treating ROS and melanogenesis, without causing any cytotoxicity in either adipocytes or melanocytes. Among the compounds being tested, MTD, labdadiene, hispidin and DK are among four potent inhibitors of ROS and NO production in 3T3-L1 adipocytes. They also reduce the melanogenesis, suggesting that a common mechanism is involved in both anti-ROS/NO production and anti-melanogenesis. Nevertheless it is clear that alpinia extracts could be utilized for treating obesity as well as a skin-whitening cosmetic agent for the treatment of hyper-pigmentation.

Regarding the detailed molecular mechanism underlying anti-melanogenesis by these alpinia compounds, several lines of indirect evidence suggest that these herbal compounds could block melanogenesis and NO production through their PAK1-blocking activity: *i*) a variety of herbal compounds such as CAPE, curcumin, and FTY720 that block the

oncogenic/ageing kinase PAK1 also strongly inhibit the melanogenesis by down-regulating the oncogenic and melanogenic transcription factor MITF which is essential for the expression of tyrosinase gene(s) (*15*); *ii*) among these alpinia compounds, at least hispidin, DK and DDK are known to inhibit directly PAK1 *in vitro* (*3*); *iii*) NO production requires PAK1 (*19*); *iv*) we have recently confirmed biochemically by shRNA-induced silencing of PAK1 gene in melanocytes that PAK1 is essential for the melanogenesis in skin cells (Be Tu *et al*., manuscript submitted). However, it is also possible that some of these alpinia compounds might block PAK4 as well, because contribution of PAK1 to the melanogenesis is estimated only 50%, and some of these compounds, in particular MTD, inhibits the melanogenesis clearly by far more than 50%. Thus, it would be worth testing the anti-PAK1/anti-PAK4 activity of MTD, labdadiene and TMOQ from alpinia seeds in cell culture. In addition, so far nobody has studied the possible role of PAK4 in either obesity or ROS/NO production in adipocytes. Perhaps it could be worth testing this possibility as well.

Finally, it might be worth noting that years ago several compounds such as galangin and kaempheride from *Alpinia officinarum* were found to inhibit the melanogenesis of melanoma cell line (*29*). However, the anti-melanogenic compounds from *Alpinia zerumbet* studied here have nothing to do with those (from *Alpinia officinarum*) previously reported. These findings indicate a surprising diversity of their ingredients among the alpinia family, and yet they share the common cosmetic/pharmacological property.

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Original Article

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Liposome encapsulated of temozolomide for the treatment of glioma tumor: preparation, characterization and evaluation

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Summary **Temozolomide plays a critical role in curing glioma at present. The purpose of this work was to develop a suitable drug delivery system which could prolong the half-life, improve the brain targeting, and reduce the systemic effect of the drug. Temozolomide-liposomes were formulated by the method of proliposomes. They were found to be relatively uniform in size of 156.70 ± 11.40 nm with a narrow polydispersity index (PI) of 0.29 ± 0.04. The average drug entrapment efficiency and loading capacity were 35.45 ± 1.48% and 2.81 ± 0.20%, respectively. The pH of temozolomide-liposomes was 6.46. *In vitro* release studies were conducted by a dynamic dialysis. The results showed that temozolomide released slowly from liposomes compared with the solution group. The release behavior of temozolomide-liposomes was in line with First-order kinetics and Weibull equation. The pharmacokinetics study was evaluated by pharmacokinetics parameters. The $t_{1/2\beta}$ and MRT of temozolomide-liposomes were 3.57 times and 1.27 times greater than that of temozolomide solution. The C_{max} and AUC values of temozolomide-liposomes were 1.10 times and 1.55 times greater than that of temozolomide solution. The results of pharmacokinetics study showed temozolomide-liposomes prolonged the *in vivo* circulation time and increased AUC. Furthermore, the biodistribution in mice showed that temozolomide-liposomes preferentially decreased the accumulation of temozolomide in heart and lung and increased the drug concentration in brain after *i.v.* injection, which implied that temozolomide-liposomes improved the therapeutic effect in the brain and reduced the toxicity in lung and heart.**

Keywords: Temozolomide, liposomes, gliomas, pharmacokinetics, biodistribution

1. Introduction

Glioma with an incidence of 5 per 100,000 persons is one of the most devitalizing malignant diseases (*1*). They are very difficult to resect entirely because of the infiltrative nature of the tumors and these tumors almost invariably recur, rapidly leading to death (*2*). Surgical resection alone is inadequate for cure, so enough effective chemotherapy drugs are desiderated.

Temozolomide is a new type of imidazole tetrazines drugs with demonstrated efficacy for patients with recurrent gliomas. During adjuvant temozolomide, the median survival of the patient with gliomas was about 16 months, and the survival rates of 1-year and 2-year were 58% and 31%, respectively (*3*). Generally, temozolomide is regarded as tolerable, effective, and well absorbed after oral administration. However, because of its short half-life of about 1.8 h in plasma, temozolomide must be administered in high systemic doses to achieve therapeutic brain levels (*4*). Furthermore, prolonged systemic administration is associated with systemic effect including nausea and vomiting, headache, and fatigue (*5*). Temozolomide injection is more suitable for post-operative patients. However, the marketable temozolomide lyophilized powder for injection used Tween 80 due to the poor solubility of the drug, which can cause hemolysis, allergies and other adverse reactions (*6,7*). Therefore, the development of new anti-glioma nano drug delivery systems became the research focuses to prolong the half-life, improve the brain targeting, and reduce the systemic effect of temozolomide. Huang *et al*. (*8*) prepared the solid lipid nanoparticles of

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temozolomide, which exhibited sustained release and brain targeting.

Liposomes as the first closed bilayer phospholipid systems were described in 1965 and they were proposed as drug delivery systems soon (*9*). Both hydrophilic drugs and hydrophobic drug can be entrapped. The significant advance was the ability to keep the integrity of the liposome structure when entrapping drugs in it. This property has been demonstrated to significantly affect the stability and pharmacokinetics of dugs (*10*). With this property liposomes can protect temozolomide from depredating in the plasma, which can improve the stability of the temozolomide and prolong considerably the temozolomide half-life in the circulation. In addition, liposome could be consumed by monocytes, neutrophils, *etc*., which can pass through blood brain barrier in the body circulation process selectively and get to the brain (*11*). Besides, it can get to the brain tissue through the blood brain barrier by pinocytosis of endothelial cells (*12,13*). So the drug concentration in the brain could increase significantly, when the temozolomide was entrapped in liposome.

The aim of this present work was to develop temozolomide-liposomes to prolong the half-life, reduce the systemic effect, and improve the brain targeting, the therapeutic efficacy and the patient compliance. In this study, temozolomide-liposomes was developed by the method of proliposomes method with the materials of soybean lecithin and cholesterol and characterized by the particle size and its distribution, pH value, morphology, drug loading, drug encapsulation efficiency, and drug release behavior *in vitro*. The *in vivo* pharmacokinetics and biodistribution of temozolomide-liposomes were evaluated to elucidate their feasibility as intravenous delivery systems.

2. Materials and Methods

2.1. *Materials*

Temozolomide was purchased from Hangzhou Hesu Chemical Technology Co., Ltd. (Zhejiang, China). Soybean lecithin (injection grade, phosphatidylcholine accounts for 95% pH 5.0-7.0) was provided by Shanghai Taiwei Pharmaceutical Co., Ltd. (Shanghai, China). Cholesterol, Sorbitol, and Mannitol were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Lactin was provided by Tianjin Guangcheng Chemical Agent Co., Ltd. (Tianjin, China). Glacial acetic acid was purchased from Tianjin Fuyu Chemical Co., Ltd. (Tianjin, China). N-caprylic alcohol, hydrochloric acid, and other reagents used were of analytical grade.

2.2. *Formulation of temozolomide-liposomes*

Temozolomide-liposome was prepared by a proliposomes method (*14,15*). Briefly, temozolomide (20 mg), soybean phospholipid (240 mg), and cholesterol (30 mg) were dissolved in methanol. Then sorbitol (1.5 g) was added with the help of ultrasonic cleaner (Kun Shan Ultrasonic Instruments Co., Ltd., Jiangsu, China). The organic solvent was removed using a RE52-98 rotary evaporator (Shanghai Yarong Instrument Co., Shanghai, China) under vacuum (0.02 MPa) with the rotate speed of 80 rpm for 30 min at 40°C. The lipid was thoroughly dried to remove residual organic solvent by placing in a vacuum oven for 5 h and then filtered through the screen (20 mesh). All products were sealed immediately in vials and stored in refrigerator at 4°C. The dried lipid was rehydrated with 15 mL of the solution of temozolomide (1 mg/mL) and obtained suspensions with ultrasound for 5 min when use.

2.3. *Measurement of particle size, and pH value*

The particle size and polydispersity index (PI) of temozolomide-liposome were determined by Delsa™ Nano C Particle Analyzer (Beckman Coulter A53878, Otsuka Blectronics Co. Ltd., USA). The pH value of temozolomide-liposome was determined with a digital pH meter (FE20, Mettler Toledo, Switzerland). Each measurement was made at least in triplicate

2.4. *Transmission election microscope (TEM) examination*

TEM observations of temozolomide-liposomes were performed by transmission electron microscope (H-7000, Hitachi, Japan). Briefly, the liposome samples were dropped onto carbon-coated grids and drawn off excess solution with a piece of filter paper. Then, the grids were immersed in 2.0% phosphomolybdic acid aqueous solution for 1 min. Finally, the grids were dried and imaged using a transmission electron microscope.

2.5. *Determination of entrapment efficiency (EE) and drug-loading rate*

The EE of liposomes was determined using the ultrafiltration technique (molecular weight cutoff 10 kDa) for separating the non-entrapped drug from liposomes (*16*). In short, 50 μL of temozolomide-liposome was mixed with 1 mL of acetonitrile, and diluted it to 10 mL with 5% acetic acid (v/v). The samples were filtered using 0.22 μm membrane filter and then subjected to high-performance liquid chromatography (HPLC). The content of total temozolomide was determined.

Briefly, 100 μL of temozolomide liposome was added in an ultrafiltration centrifuge tube and mixed with 700 μL of distilled water. The free temozolomide was separated from liposome by ultrafiltration at 3,500 rpm for 30 min. After completed the centrifugation, 200 μL of distilled water was added and shake, then centrifuge again by ultracentrifugation at 3,500 rpm

for 20 min. Suck up 50 μL of filtrate and dilute to 10 mL with 5% acetic acid (v/v). The samples were filtered using 0.22 μm membrane filter and injected into the HPLC. The content of free temozolomide was determined.

$$EE\% = \frac{W_{total} - W_{free}}{W_{total}} \times 100\% \quad (1)$$

$$DL\% = \frac{W_{total} - W_{free}}{W_{lipid}} \times 100\% \quad (2)$$

Where W_{free} was the analyzed weight of free drug, W_{total} was the analyzed weight of drug in liposomes, W_{lipid} was the analyzed weight of liposomes.

2.6. *HPLC analysis*

The HPLC equipment was a Shimadzu LC-10A system (Shimadzu, Kyoto, Japan) consisting of a LC-10AT HPLC pump and a SPD-10A UV-VIS detector. Data processing was performed with LC Solution software. Drug analysis was conducted on a Diamonsil-ODS column (150 × 4.6 mm, Dikma Technologues, China). The mobile phase consisted of methanol, deionized water, and glacial acetic acid (10:89.5:0.5, v/v) at a flow rate of 1.0 mL/min and an injection volume was 20 mL. The detection wavelength was set at 329 nm

2.7. *In vitro release study*

In vitro release rate behaviors of temozolomide from liposome vesicles were performed by the dialysis method. Briefly, 3 mL of liposome suspension (the concentration is 1 mg/mL) was loaded in dialysis bag (molecular weight cutoff 8 to 14 kDa) and dialyzed against the release media of PBS with pH 5.0 at 37 ± 0.5°C in an water bath shaker at 100 rpm. At scheduled time (0.083, 0.17, 0.33, 0.5, 0.75, 1, 2, 4, and 6 h) intervals, 0.2 mL of the release media was collected and then the same volume of fresh release media were added. Dilute the solution to 1 mL with 5% acetic acid (v/v). The release amount of temozolomide was determined by HPLC as described earlier. The mean calculated values were obtained from 3 replicates. The drug release profile of temozolomide was examined as a control. The accumulative release percentage of temozolomide (R%) was calculated according to the following equation:

$$R = \frac{c_n v_0 + \sum_{i=0}^{n-1} c_i v_i}{W} \times 100\ \%$$

where R is the release rate, c_n is the drug concentration in the release medium of each time interval, v_0 is the total volume of the release medium, v_i is the volume of the withdrawn medium, c_i is the drug concentration in the release medium at time, and W is the total drug content of the release sample.

2.8. *Pharmacokinetics studies in rabbits*

The *in vivo* pharmacokinetics experiment was carried out with adult white New Zealand rabbits (female and male) weighing 2.0-2.5 kg supplied by the Medical Animal Test Center of Shandong University. The animal experiment protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Shandong University. The rabbits used for this study were housed individually under normal conditions, and fasted overnight before experiment with free access to water. Animals were randomly assigned into two groups with six rabbits each. Rabbits in one group were administered with temozolomide liposomes (equal to 10 mg/kg temozolomide) by injection in one of the ear marginal vein, while control group was administered with 10 mg/kg temozolomide solution (5 mg/mL, dissolved in physiological saline solution). Blood was collected at specified time intervals (0.083, 0.167, 0.333, 0.5, 1, 2, 3, 5, 8, 10, 12 h) by intracardiac puncture and was put into centrifuge tube washed with EDTA-2Na (15 mg/mL) along with 10 μL of a 10% phosphoric acid solution. Plasma samples were harvested by centrifuging at 12,000 rpm for 10 min. Subsequently, 300 μL of the resulting rabbit plasma was added to 300 μL protein precipitation (10% trichloroacetic acid:acetonitrile = 3:2) and the mixture was vortexed for 30 sec. The sample was centrifuged for 10 min at 12,000 rpm. After centrifugation, the supernatant layer was filtered through a 0.22 μm filter, and then 20 μL of the filtered solution was directly injected into HPLC for analysis.

The plasma samples were determined by the HPLC system. The HPLC condition was the same as described above except for the mobile phase consisted of methanol, deionized water, and glacial acetic acid (8:91.5.5:0.5, v/v).

Method validation: weigh 61.2 mg of temozolomide, then dissolve and dilute to 50 mL with water. Put the temozolomide solution as stock solution. Pipe appropriate amount stock solution to prepare a series of concentration of temozolomide (1.5, 3, 30, 150, 306, 612, 1,224 μg/mL). Pipe 10 μL of the series of concentration of temozolomide solution respectively, then add 0.28 mL of plasma and 10 μL of 10% phosphoric acid solution (w/v), mix well. Then a series of concentration of temozolomide (0.051, 0.1, 1.02, 5.1, 10.2, 20.4, 40.8 μg/mL) were obtained. Dispose the plasma with temozolomide with the method as above. Injected 20 μL sample and record the peak area (A). Draw the standard curve and calculate the CV% and recovery.

2.9. *Drug distribution studies in mice*

Kunming strain mice (weighed between 18 and 22 g, female or male were provided by the Medical Animal Test Center of Shandong University) were used for the biodistribution studies. The animals were fasted 12 h

before drug administration. The animal experiment protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Shandong University.

Two groups of 15 mice each were used for the *in vivo* distribution studies. temozolomide-liposomes and temozolomide solution were injected through the tail veins of mice at a dosage of 20 mg/kg. Following drug administration, at each predetermined time point (0.083, 0.25, 0.5, 1, 2 h), three mice in each group were then sacrificed by cervical dislocation, and the spleen, kidneys, liver, brain, heart, and lungs were surgically removed. Each organ sample was washed with physiological saline solution, and the redundant liquid was blotted using absorbent paper. Accurately weighted tissue specimen was homogenized with 1 mL physiological saline solution (except for liver, 2 mL) by homogenizer. Tissue homogenates were processed as the plasma sample. After centrifugation, the supernatant was passed through a 0.22 μm filter. About 20 μL of the filtered tissue homogenate was injected into HPLC for analysis. The HPLC condition was the same as described in Pharmacokinetics studies in rabbits.

3. Results and Discussions

3.1. *Physicochemical characterization of the liposome*

The photographs of three batches of the formulation were shown in Figure 1. The appearance of liposomes after rehydration is semitransparent with opalescence.

The particle size, polydispersity index, entrapment efficiency, drug loading, and pH of three batches of the formulation were reported in Table 1. The mean particle size of the formulation was 156.7 ± 11.4 nm. Furthermore, the PI of temozolomide-liposomes was 0.290 ± 0.041, which revealed that the formulation had a quite narrow size distribution and uniform size. The mean pH values of temozolomide-liposomes was 6.46 ± 0.08, which was within the safe pH scope of 4-9 for intravenous injection. The mean drug entrapment efficiency and drug loading was 35.45 ± 1.48% and 2.81 ± 0.20%, respectively. The size distribution of temozolomide-liposomes shown in Figure 2. The results indicated that singe-peak and narrow distribution were obtained.

To obtain more information about the morphology of temozolomide-liposomes, TEM analysis was performed and the result of TEM image was showed in Figure 3. The image displayed that temozolomide-liposomes had relatively spherical shapes and had obvious hydrophilic layer, which may be due to sorbitol covering in the surface of the liposome. The particle

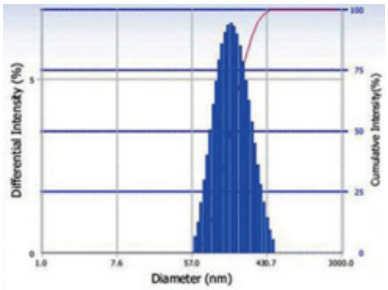

Figure 2. The size distribution of temozolomide liposomes. The results indicated that singe-peak and narrow distribution were obtained.

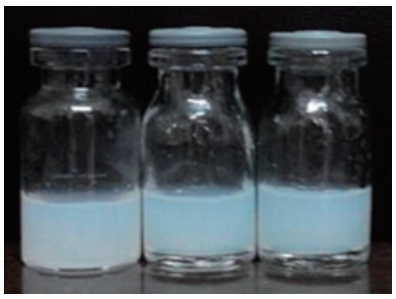

Figure 1. Appearance of liposomes after rehydration. The photographs of three batches of the formulation were shown. The appearance of liposomes after rehydration is semitransparent with opalescence.

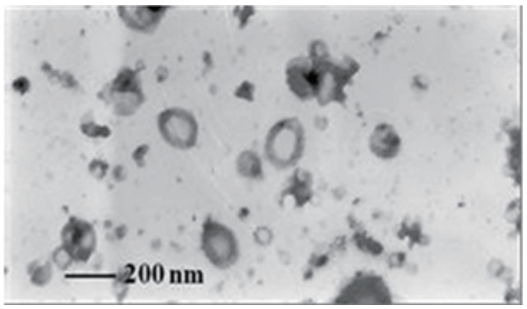

Figure 3. Transmission electron microscopy micrographs of temozolomide. The image displayed that temozolomide-liposomes had relatively spherical shapes and had obvious hydrophilic layer, which may be due to sorbitol covering in the surface of the liposome.

Table 1. The particle size, polydispersity index, entrapment efficiency, drug loading and pH of three batches of the formulation

Batches	Particle size (nm)	PI	EE (%)	DL (%)	pH
1	159.4	0.334	36.40	2.70	6.49
2	144.2	0.282	33.75	2.68	6.53
3	166.5	0.254	36.21	3.04	6.37
$\bar{\chi}$ ± S.D.	156.7 ± 11.4	0.290 ± 0.041	35.45 ± 1.48	2.81 ± 0.20	6.46 ± 0.08

PI, polydispersity index; EE, entrapment efficiency; DL, drug loading.

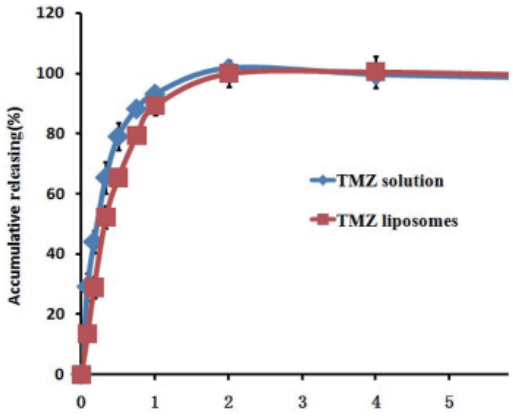

Figure 4. *In vitro* release profile of temozolomide from temozolomide solution and temozolomide-liposomes in phosphate-buffered saline (pH 5.0) at 37 ± 0.5°C (*n* = 3). The release behavior of temozolomide- liposomes was in accordance with the Weibull equation and First-order kinetics.

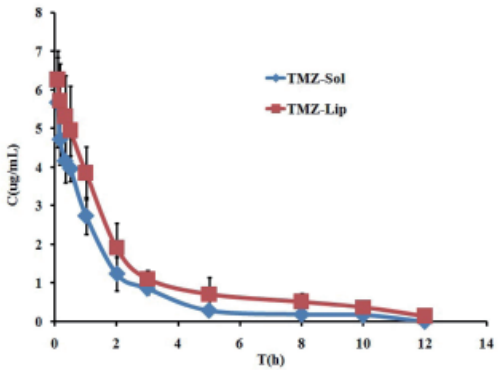

Figure 5. The temozolomide concentration-time curve after intravenous administration of temozolomide solution and temozolomide- liposomes in rabbits (*n* = 6). The plasma temozolomide concentration of temozolomide-liposomes group was much higher than that of the temozolomide solution group at each sampling time.

size determined by TEM agreed well with the results from Delsa™ Nano CParticle Analyzer.

3.2. *In vitro release behaviors of temozolomide-liposomes*

The release behavior profile of temozolomide-liposomes and temozolomide solution *in vitro* was shown in Figure 4. The release behavior of temozolomide-liposomes was in accordance with the Weibull equation and First-order kinetics. It can be expressed using the following equation: $\ln\ln(1/(1 - Q/100)) = 1.0805\ln t + 0.8133$, $r = 0.9988$ and $\ln(100 - Q) = -2.2329t + 4.6426$, $r = 0.9988$. The release behavior of temozolomide solution was in accordance with the Weibull equation model and can be expressed using the following equation: $\ln\ln(1/(1 - Q/100)) = 0.8419\ln t + 1.0014$, $r = 0.9992$. The results of *in vitro* release profile of temozolomide from temozolomide solution and temozolomide-liposomes were shown that temozolomide was released slower from temozolomide-liposomes than temozolomide solution at first. The slow release characteristic at this phase could be attributed to the fact that temozolomide was held by the lipid and therefore temozolomide was released gradually from the lipid matrices mainly through dissolution and diffusion (*17*). However, after 2 h, the release rate of the drug from the liposomes and solution were both observed around 100% and their release behaviors have no obvious difference. This phenomenon may be explained by the existing of the free drugs in the preparations because of the low encapsulation efficiency.

3.3. *Pharmacokinetics study in rabbits*

At the range of 0.051-40.8 μg/mL, the standard curve was $A = 32.423C - 2.6433$ and the standard showed a good linearity with a correlation coefficient of 0.9999. The CV% of is no more than 6% and the recovery is no less than 93%. The mean drug concentration in plasma versus time profile after the intravenous administration of liposomes and suspension was shown in Figure 5, and the pharmacokinetic parameters calculated by DAS 2.0 software were summarized in Table 2. After *i.v.* administration, the plasma temozolomide concentration of temozolomide-liposomes group was much higher than that of the temozolomide solution group at each sampling time. Furthermore, temozolomide-liposomes were eliminated more slowly than temozolomide solution. Besides, following injection of temozolomide-liposomes, temozolomide concentration was still measured to be 0.37 mg/L after 10 h, while temozolomide concentration of temozolomide solution was detected to be 0.29 mg/L after 5 h. Compared with temozolomide solution, the pharmacokinetic parameters of liposome group have been changed: $t_{1/2\beta}$ and MRT were 13.312 h and 3.402 h, which were 3.57 times and 1.27 times of temozolomide solution; C_{max} and AUC values of temozolomide-liposomes were 6.26 mg/L and 16.158 mg/L·h, which were 1.10 times and 1.55 times of temozolomide solution. The results of pharmacokinetics study showed that prolonged *in vivo* circulation time and increased AUC were in favor of temozolomide treatment for gliomas. These results could account for the rapid distribution and metabolism

Table 2. The pharmacokinetic parameters of temozolomide after *i.v.* injection of temozolomide solution and temozolomide-liposomes in rabbits (*n* = 6)

Parameters	Temozolomide -Solution	Temozolomide -Liposomes
t1/2α (h)	0.745	1.050
t1/2β (h)	3.731	13.312
V1 (L/kg)	1.741	1.531
CL (L/h/kg)	0.956	0.619
AUC0-t (mg/L*h)	10.457	16.158
MRT	2.686	3.402
Tmax (h)	0.083	0.083
Cmax (mg/L)	5.680	6.260

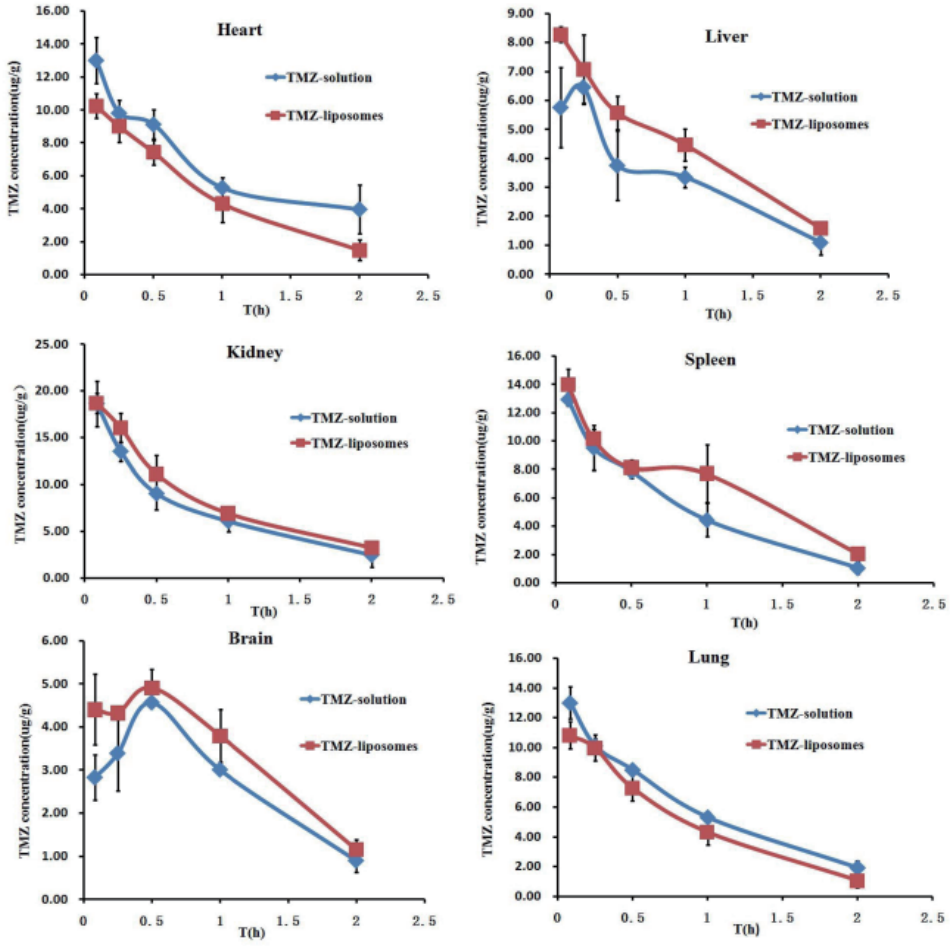

Figure 6. Temozolomide concentration-time curve in tissue at different time points after intravenous (*i.v.*) administration of temozolomide-solution and temozolomide-liposomes ($n = 3$). The biodistribution profiles of temozolomide in mice after intravenous administration of temozolomide solution and temozolomide-liposomes were shown. Briefly, the drug concentrations in brain were elevated after the administration of temozolomide-liposomes compared with the temozolomide solution. Meanwhile, the drug concentration in liver, kidney and spleen increased but decreased in heart and lung after administration of the liposomal formulation compared with temozolomide solution.

of the free temozolomide, compared to the distribution of temozolomide entrapped in liposomes. This might be explained by the protection of the lipid bilayer membranes and slow drug release from liposomes (*18*), which was also shown in the *in vitro* release experiment. At the same time a water shield on the surface of the liposomes reduced adsorption of opsonins, thereby reducing the intake of mononuclear phagocytes. That was one of the reasons why temozolomide-liposomes could remain in the circulation for a longer period of time than temozolomide solution.

3.4. *Drug distribution studies in mice*

The biodistribution profiles of temozolomide in mice after intravenous administration of temozolomide solution and temozolomide-liposomes were shown in Figure 6. Some changes of distribution can be observed between temozolomide solution and liposomes after the intravenous administration. Briefly, the drug concentrations in brain were elevated after the administration of temozolomide-liposomes compared with the temozolomide solution which could improve the efficiency of temozolomide treatment for gliomas. Meanwhile, the drug concentration in liver, kidney and spleen increased but decreased in heart and lung after administration of the liposomal formulation compared with temozolomide solution, which may be expected to reduce the potential side effects in heart and lung (*19*). The increasing concentration of temozolomide in liver and spleen may be explained by the fact that liposomes < 200 nm tend to be taken up by macrophages in liver and spleen more easily. The reason resulted to the increasing concentration of temozolomide in kidney was liposomes tend to be into the kidney easily due to the hydrophilic layer of sorbitol on the surface of liposomes (*20*). Furthermore, temozolomide-liposomes resulted in a higher drug accumulation in brain compared with temozolomide solution, which may be explained by the fact that the RES removal of the nanoparticles can be prevented by surface coating with hydrophilic polymers to increase their availability at brain (*21*). Liposome could be consumed by monocytes, neutrophils, *etc.*, which can pass through blood brain barrier in the body

Table 3. Tissue targeting parameters of temozolomide solution and temozolomide liposomes after *i.v.* administration in mice (n = 6)

Tissues	AUC		Re
	Temozolomide-Sol	Temozolomide-Lip	
Heart	13.58	10.366	0.763
Liver	6.812	9.106	1.337
Spleen	10.975	14.328	1.306
Lung	12.444	10.426	0.838
Kidney	15.178	17.494	1.153
Brain	5.656	6.9	1.220

Re = AUCliposomes/AUCsolution.

circulation process selectively and get to the brain. Besides, it can get to the brain tissue through the blood brain barrier by pinocytosis of endothelial cells. So the drug concentration in the brain could increase significantly. At the same time a water shield on the surface of the liposomes reduce adsorption of opsonins, thereby reducing the passive targeting to lung.

To represent targeting efficiency, AUC and the targeting parameters such as the intake rate (Re), were calculated and are listed in Table 3. temozolomide-liposomes exhibited a larger value of AUC for brain compared with temozolomide solution. The results showed that compared to temozolomide solution, Re value of temozolomide-liposomes in the brain was 1.22, Re values in the liver and spleen were 1.337 and 1.306, Re values in the kidney was 1.153, and Re values in the heart, lungs were 0.763 and 0.838, which also implied that temozolomide-liposomes increased the concentration of the drug in the brain, improved the therapeutic effect and reduced the toxicity in heart and lung.

In this study, temozolomide-liposomes were prepared for intravenous injection with the method of proliposomes successfully. The preparation process was simple with good reproducibility, and temozolomide-liposomes possess displayed excellent performance featured by small and uniform size. The pH was within the safe pH scope of 4-9 for intravenous injection. The results of *in vitro* drug release behavior showed that temozolomide-liposomes released slowly compared with temozolomide solution. The results of pharmacokinetics and tissue distribution study showed that the liposomes had a slow-release effect and brain targeting. Furthermore, temozolomide-liposomes could reduce the C_{max} in some organs such as heart, lung potentially decreasing the side effects. temozolomide-liposomes can change biodistribution, increase the concentration of drug at the treatment site, and reduce side effects in non-target tissues. Furhermore temozolomide- liposomes avoided the use of Tween 80 which was contained in the listed freeze-dried powder for injection which might be helpful to decrease the side effects. The drug delivery system can prolong the half-life, improve the targeting and reduce the systemic effect of the drug, which were promising for developing the new formulation. The following work would be focused on optimizing prescription and process to improve the encapsulation efficiency.

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Original Article

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Preparation and evaluation of gelling granules to improve oral administration

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Summary **We investigated the preparation of oral granules that are solid when stored and that will swell and gel *via* water absorption, to address problems experienced by patients when taking medication. Important physical properties of gelling granules include elasticity that is normally smooth, quick water absorption and swelling properties that allow easy swallowing. We selected gelatin (GEL), succinylated gelatin (SUC-GEL) and ι-carrageenan (CAR) as matrix polymers that can undergo gelation at room temperature or at cold temperatures. Saccharide and polyethylene glycol (PEG) were added to prepare the experimental granules. The best matrix gelling granule was SUC-GEL. When xylitol (XYL), sorbitol (SOR) and maltitol (MAL) were added, elasticity was improved, and PEG improved the granule's water absorption behavior, which is an important element involved in gelation. The best granules were prepared by selecting SUC-GEL as the matrix and adding a small amount of PEG and XYL in amounts equal to that of SUC-GEL.**

Keywords: Polymer, gelling granules, succinylated gelatin, saccharide, polyethylene glycol

1. Introduction

Oral preparations are widely used, and they are an important type of formulation in pharmacotherapy. Any issue that the patient has when they take their medication affects the curative effect by reducing the patient's compliance or adherence. Tablets are the most preferred form of oral preparations, and they are frequently used because of their handling convenience. However, there are several problems with taking tablets, which can cause difficulties for infants and patients with difficulties swallowing (*1-4*). Therefore, powder, granules, dry syrups, and syrup are often given to these patients. These formulations cause resistance that results from discomfort when taking the medicine, because they can spread and residue can remain in the mouth or feel rough. Addressing these problems that might lead to improvement of the curative effect and quality of life (QOL) for the patient. To date, a jelly preparation (*5-7*), orally disintegrating tablets (*8-11*) and an oral film preparation have been developed to improvement administration. However, the content of these preparations is constant, similar to tablets, which represents a difficulty for children or elderly people who need to adjust the dose. We aimed to determine a new, adjustable preparation with granules that will help improve patient compliance. We targeted properties such as a solid form when stored and the ability to swell and gel quickly by absorbing water for comfortable swallowing. There are many reports of preparations that gel and swell by absorbing water (*12,13*). However, this feature has not often been reported to help improve the patient's experience when they take medication (*14,15*), and there have been no previous reports that have involved the use of granules. In this study, we focus on the properties of xelogel, which changes to hydrogel when water is added, and we investigated gelling granules that are dry until the patient drinks it with water.

2. Materials and Methods

2.1. *Materials*

Acetaminophen (AA) was purchased from Sigma-Aldrich (St. Louis, MO., USA) as model drug. Gelatin (GEL, Japanese Pharmacopoeia (*21*),Tokyo, Japan)

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was obtained from Nacalai Tesque (Kyoto, Japan) and succinylated gelatin (SUC-GEL) was obtained from Higuchi (Tokyo, Japan). ι-Carrageenan (CAR) was obtained from Sigma-Aldrich as a polymer. Sorbitol (SOR) and maltitol (MAL) were obtained from Towa Chemical Industry Co., Ltd. (Tokyo, Japan), and xylitol (XYL), mannitol (MAN) and polyethylene glycol (PEG) were obtained from Nacalai Tesque. Magnesium stearate (stMg) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) to be used as the hydrophobic powder material upon which granules were prepared.

2.2. *Granule preparation*

Polymer and AA were dissolved in water solution at 60-70°C in a hot water bath. Using a micropipette, the solution was dropped onto stMg and allowed to spread all over the tray. The granules were then dried in a refrigerator. We obtained the granules by removing excess stMg. Formulation of several granules prepared was shown in Table 1-4.

2.3. *Granule evaluation*

Granules of 1-2 mm were selected for further testing.

2.3.1. *Granule strength*

The granule strength was measured using a rheometer (Sun Scientific Co. Ltd., Tokyo, Japan). Using an adapter that was 10 mm in diameter, granules were placed on the sample table, which placed them under pressure by raising the sample table at a speed 15 mm/min. Thus, the displacement and load were obtained (n = 5).

2.3.2. *Water absorption test*

Granule water absorption behavior was evaluated by measuring the increase in granule weight when the granules were placed in a mesh basket after contact with a known amount of purified water. The increased weight ratio was obtain by measuring the amount of water absorption every 30 sec (n = 5).

2.3.3. *Dissolution study*

The paddle method, using the JP 16 dissolution apparatus (Toyama Sangyo Co. Ltd., Osaka, Japan), was used in this experiment. The dissolution medium was 900 mL purified water at 37°C, with a stirring rate of 100 rpm. At appropriate time intervals, a quantity of samples were withdrawn and replaced with the same volume of purified water. These samples were diluted with purified water as needed. AA concentrations were determined using UV spectrophotometry (Shimadzu Co., Kyoto, Japan) at a wavelength of 244 nm (n = 3).

Table 1. Formulation of granules prepared with polymer and saccharides

Material	GEL/MAN	GEL/XYL	GEL/SOR	GEL/MAL	SUC-GEL/MAN	SUC-GEL/XYL	SUC-GEL/SOR	SUC-GEL/MAL	CAR/MAN	CAR/XYL	CAR/SOR	CAR/MAL
Acetaminophen (g)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.5	0.5	0.5	0.5
Gelatin (g)	5.0	5.0	5.0	5.0	-	-	-	-	-	-	-	-
Succinylated gelatin (g)	-	-	-	-	5.0	5.0	5.0	5.0	-	-	-	-
ι-Carrageenan (g)	-	-	-	-	-	-	-	-	1.0	1.0	1.0	1.0
Mannitol (g)	5.0	-	-	-	5.0	-	-	-	1.0	-	-	-
Xylitol (g)	-	5.0	-	-	-	5.0	-	-	-	1.0	-	-
Sorbitol (g)	-	-	5.0	-	-	-	5.0	-	-	-	1.0	-
Maltitol (g)	-	-	-	5.0	-	-	-	5.0	-	-	-	1.0
Water (mL)	q.s.											
Total (mL)	100											

Values are given as volume per 100 mL. AA concentration of granules was prepared as a 20% (200 mg/g). q.s., quantum sufficiat; AA, acetaminophen; GEL, gelatin; SUC-GEL, succinylated gelatin; CAR, ι-carrageenan; MAN, mannitol; XYL, xylitol; SOR, sorbitol; MAL, maltitol.

Table 2. Formulation of granules prepared with CAR and SUC-GEL and saccharides

Material	GrA	GrB	GrA/XYL	GrA/MAL	GrB/XYL	GrB/MAL
Acetaminophen (g)	1.25	1.25	2.5	2.5	2.5	2.5
Succinylated gelatin (g)	4.5	4.0	4.5	4.5	4.0	4.0
ι-Carrageenan (g)	0.5	1.0	0.5	0.5	1.0	1.0
Xylitol (g)	-	-	5.0	-	5.0	-
Maltitol (g)	-	-	-	5.0	-	5.0
Water (mL)				q.s.		
Total (mL)				100		

Values are given as volume per 100 mL. AA concentration of granules was prepared as a 20% (200 mg/g). q.s., quantum sufficiat; AA, acetaminophen; CAR, ι-carrageenan; SUC-GEL, succinylated gelatin; XYL, xylitol; MAL, maltitol.

Table 3. Formulation of granules prepared with CAR and SUC-GEL and saccharides

Sample	Acetaminophen (g)	Succinylated gelatin (g)	Polyethylene glycol-400 (mL)	Xylitol (g)	Water (mL)	Total (mL)
SUC-GEL/PEG(1)	1.5	5.0	1.0	-		
SUC-GEL/PEG(2)	1.8	5.0	2.0	-		
SUC-GEL/PEG(3)	2.1	5.0	3.0	-	q.s.	100
SUC-GEL/PEG(10)	4.1	5.0	10.0	-		
SUC-GEL/XYL	2.5	5.0	-	5.0		

Values are given as volume per 100 mL. AA concentration of granules was prepared as a 20% (200 mg/g). q.s., quantum sufficiat; AA, acetaminophen; SUC-GEL, succinylated gelatin; PEG, polyethylene glycol-400; XYL, xylitol.

Table 4. Formulation of granules prepared with SUC-GEL and PEG and XYL

Sample	AA (g)	SUC-GEL (g)	PEG (mL)	XYL (g)	Water (mL)	Total (mL)
SUC-GEL/PEG(1)/XYL	2.8	5.0	1.0	5.0	q.s.	100
SUC-GEL/PEG(10)/XYL	5.3	5.0	10.0	5.0		

Values are given as volume per 100 mL. AA concentration of granules was prepared as a 20%. AA, acetaminophen; SUC-GEL, succinylated gelatin; PEG, polyethylene glycol-400; XYL, xylitol.

2.3.4. *Statistical analysis*

The results are presented the mean ± standard deviation (S.D.). Statistical differences were analyzed using the Tukey-Kramer test for multiple comparisons, and the level of significance was set at $p < 0.05$.

3. Results

3.1. *Influence of polymer and saccharide type*

Composition of each granule that used a polymer of GEL, SUC-GEL and CAR as the matrix in addition to four saccharides are shown Table 1. The AA content was prepared at 20% (200 mg/g). Concentration of the polymer was 5% (w/v) for the GEL and SUC-GEL, and 1% (w/v) for CAR. These granules were mixed with various saccharides at a ratio of 1:1.

3.1.1. *Granule strength*

A strength test for each polymer yielded a stress-displacement curve (Figure 1). In any polymer containing GEL, SUC-GEL or CAR, granules that contained MAN had the most variation in load because the compression power changed on the stress-displacement curve, which showed that they were inelastic. However, granules containing XYL, SOR or MAL with GEL and SUC-GEL showed little variation. There was no difference observed among the granules, but they showed elasticity. When CAR was used for the matrix, granules elasticity tended to increase with XYL and SOR. Compared to granules using each polymer containing MAL, XYL and SOR without MAN, which had no effect on elasticity, there were no differences in the type of polymer between XYL and SOR (Figure 1). However, granules that contained MAL with GEL and SUC-GEL had a tendency to increase in elasticity.

3.1.2. *Granule water absorption*

Water absorption behavior was shown by a relationship between the square root of time (seconds) and the rate at which weight increased for each polymer (Figure 2). For GEL and SUC-GEL, the difference depended on the type of saccharide. GEL granules showed a tendency to increase the amount of water absorbed when MAL was added. The findings were similar with SUC-GEL granules when XYL was added. The CAR granules showed no difference for the various saccharides. The order of increasing weight ratio compared for each polymer is as follows: CAR > SUC-GEL > GEL. This showed that a large amount of water was absorbed when CAR was used.

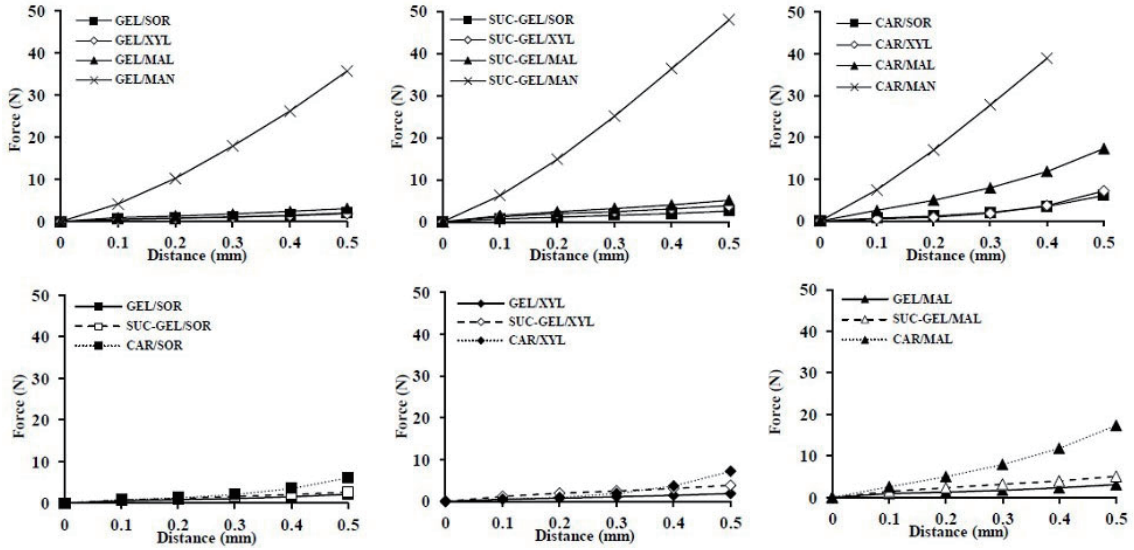

Figure 1. Elasticity of granules prepared with polymer and saccharides.

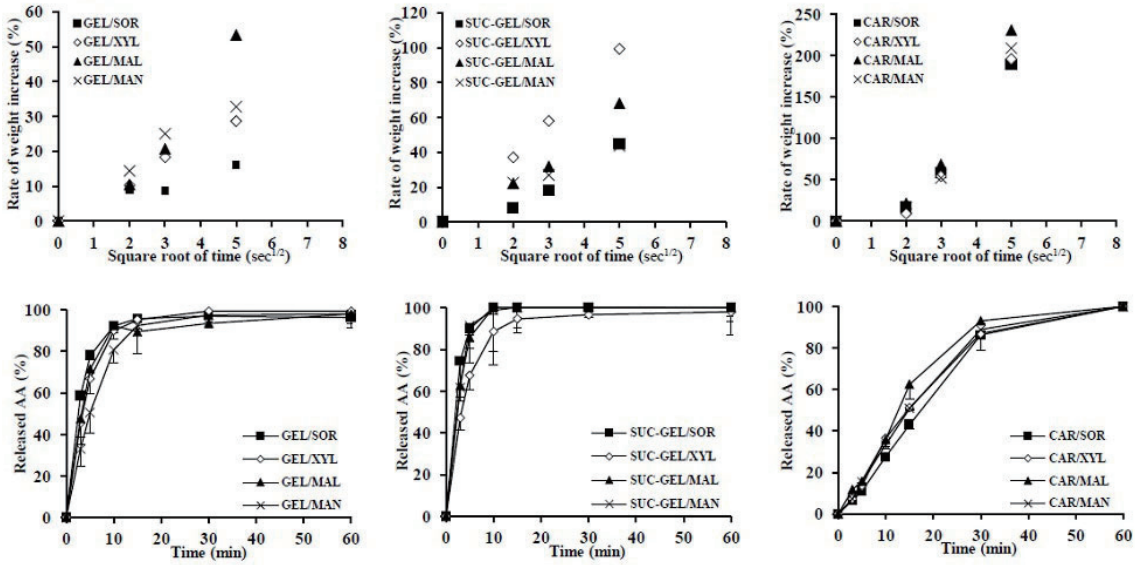

Figure 2. Water absorption and dissolution profiles of granules prepared with polymer and saccharides. Values are mean ± S.D. (n = 3).

The apparent water absorption speed relationship was a straight-line slope with the square root of time (seconds) and the rate of weight increase. Granules with GEL and SUC-GEL had a straight-line relationship (R^2 = 0.896-0.997) and the slope (apparent rate coefficient) was 3.0-18.0 (Table 5). This shows that SUC-GEL with XYL was the fastest (18.0) followed by SUC-GEL with MAL (11.6). CAR showed an apparent rate coefficient of 27.99-33.9, which was apparently larger than GEL and SUC-GEL. However, the straight-line relationship was not shown in any granules that included CAR (R^2 = 0.761-0.805). From Figure 2, there was a small amount of water absorption behavior initially, which increased thereafter.

3.1.3. *Granule dissolution behavior*

Dissolution behavior of AA released from the granules of each polymer is shown Figure 2. There were no differences in the type of saccharides on any polymer. Granules with either GEL or SUC-GEL released 100% of the AA at about 15 min. Granules with CAR showed a gradual dissolution behavior, which had a dissolution rate of 40-60% at 15 min and 100% at 60 min. However, this was slower than that for GEL and SUC-GEL.

3.2. *Effects of mixing polymer and saccharide*

From the results obtained by influencing the type of polymer and saccharide that affected granule elasticity and water absorption behavior, we examined the effect using mixed material of SUC-GEL and CAR to take into consideration both of these benefits. The saccharides selected were XYL and MAL. Composition of each granule is shown Table 2. The concentration of

the polymer solution was 5% (V/W) and the AA content was 20% (200 mg/g).

3.2.1. *Granule strength*

The stress-displacement curve obtained from the strength test is shown Figure 3A). Granule elasticity increases by adding saccharide regardless of the SUC-GEL and CAR mixture ratio. The polymer mixture ratio did not have an effect on elasticity.

3.2.2. Granule water absorption

Weight change in the water absorption test and the relationship between the square root of time (seconds) and the rate of weight increase is shown in Figure 3B).

Table 5. Apparent water absorption rate and linear correlation coefficient of granules prepared with polymer and saccharides

Sample	Apparent water absorption rate (Slope)	Linear correlation coefficient (R^2)
GEL/MAN	6.5	0.953
GEL/XYL	5.3	0.988
GEL/SOR	3.0	0.955
GEL/MAL	8.4	0.900
SUC-GEL/MAN	8.3	0.979
SUC-GEL/XYL	18.0	0.998
SUC-GEL/SOR	7.1	0.896
SUC-GEL/MAL	11.6	0.976
CAR/MAN	- (29.9)	0.766
CAR/XYL	- (28.1)	0.761
CAR/SOR	- (27.9)	0.805
CAR/MAL	- (33.9)	0.800
GrA	- (24.49)	0.798
GrB	- (16.19)	0.735
GrA/XYL	- (28.74)	0.780
GrA/MAL	- (21.67)	0.842
GrB/XYL	- (26.25)	0.796
GrB/MAL	- (16.88)	0.790
SUC-GEL/PEG(1)	58.3	0.987
SUC-GEL/PEG(2)	62.7	0.978
SUC-GEL/PEG(3)	58.0	0.975
SUC-GEL/PEG(10)	54.5	0.995
SUC-GEL/PEG(1)/XYL	56.8	0.990
SUC-GEL/PEG(10)/XYL	65.3	0.978

GEL, gelatin; SUC-GEL, succinylated gelatin; CAR, ι-carrageenan; MAN, mannitol; XYL, xylitol; SOR, sorbitol; MAL, maltitol; PEG, polyethylene glycol.

The slopes are shown in Table 5. There were some differences in the amount of water absorbed by each granule, but these changes were linear (R^2 = 0.735-0.842). The amount of water absorbed was large, but the speed of water absorption was initially slow for the SUC-GEL and CAR mixtures as well as for CAR alone.

3.2.3. *Granule dissolution behavior*

There were no obvious differences between the granules. The dissolution ratio was 30-60% at 15 min and 100% at 30 min. This was the same behavior as for CAR alone (Figure 3C).

3.3. *Effects of adding PEG and XYL*

Based on results from the type of polymer and saccharide, we selected SUC-GEL as the polymer, and investigated effects of adding various amount of PEG that was using as a plasticizer or as a solubilizing agent by comparing it with granules where XYL was added (Table 3).

3.3.1. *Granule strength*

The stress-displacement curve obtained from the granule strength test when the amount of PEG was varied and XYL was added is shown in Figure 4A). Granules that were mixing SUC-GEL with XYL in a ratio of 1:1 (SUC-GEL/XYL) had the highest elasticity. For granules that included PEG, the elasticity increased with an increasing amount of PEG.

3.3.2. *Granule water absorption*

The weight change in of the water absorption test showed a relationship between the square root of time (sec) and the rate of weight increase (Figure 4B). Granules that included PEG absorbed a greater amount of water and also absorbed it more rapid than granules with XYL, but there were no differences amount of PEG. The slope and linearity were obtained as the apparent water absorption speed from the relationship between the square root of time and the rate of weight increase (Table 5). All of the granules have linearity,

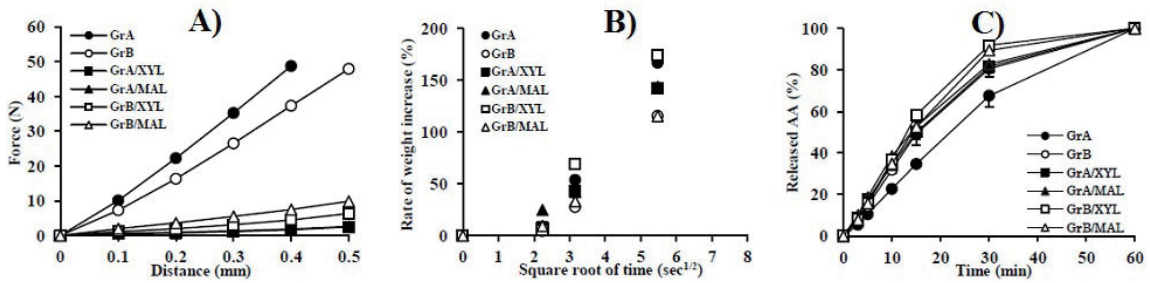

Figure 3. Characteristics of granules prepared with SUC-GEL and CAR and saccharides. A) Elasticity. **B)** Water absorption. **C)** Dissolution profiles. Values are mean ± S.D. (n = 3).

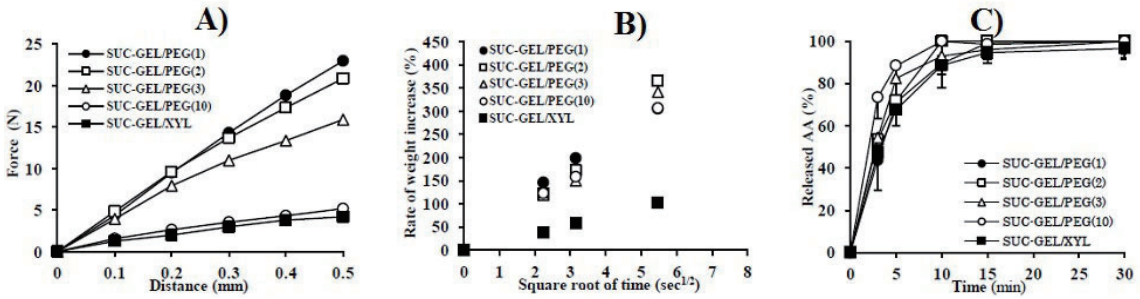

Figure 4. Characteristics of granules prepared with SUC-GEL and PEG or XYL. A) Elasticity. **B)** Water absorption. **C)** Dissolution profiles. Values are mean ± S.D. (n = 3).

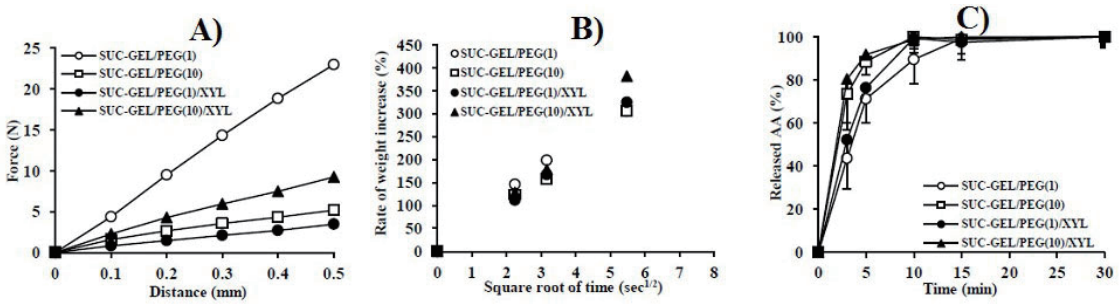

Figure 5. Characteristics of granules prepared with SUC-GEL and PEG and XYL. A) Elasticity. **B)** Water absorption. **C)** Dissolution profiles. Values are mean ± S.D. (n = 3).

and their R^2 value was greater than 0.97. The apparent water absorption speed for SUC-GEL/XYL was 18.0, and granules that included PEG were 54.5-62.7, which shows a high speed regardless of the amount of PEG that was added.

3.3.3. *Granule dissolution behavior*

There were no obvious differences in dissolution for the amount of PEG added, and comparison with granules that included XYL showed no differences. All the granules show a dissolution ratio of about 100% at 15 min (Figure 4C).

3.4. *Effect of XYL added to granules containing PEG*

When granules with various amounts of PEG and granules that included XYL were compared to SUC-GEL granules that were including 1% [V/V] of PEG (SUC-GEL/PEG (1)) and SUC-GEL granules that were including 10%[V/V] of PEG (SUC-GEL/PEG (10)), which are shown in Table 3, there were differences in elasticity. We investigated adding XYL to these granules (Table 4).

3.4.1. *Granule strength*

SUC-GEL/PEG (1), which had low elasticity, showed an increase in elasticity when XYL was added. However, SUC-GEL/PEG (10) that had high elasticity and adding XYL tended to slightly decrease this elasticity (Figure 5A).

3.4.2. *Granule water absorption*

The relationship between the square root of time (sec) and the rate of weight increase is shown in Figure 5B). There was no obvious difference and all granules showed the same water absorption behavior. Apparent water absorption speed of the granules containing XYL compared to SUC-GEL/PEG (1) and SUC-GEL/PEG (10) was 56.8 and 65.3, respectively, and effect of adding XYL was also not shown (Table 5).

3.4.3. *Granule dissolution behavior*

There were no obvious changes in dissolution behavior when XYL was adding. Any granules showed about 100% dissolution at 15 min (Figure 5C).

4. Discussion

We investigated preparation of orally-administered granules that are solid when stored and that will swell and gel when they absorb water. This research was aimed at addressing problems experienced by patients when taking medications. Required physical properties of gelling granules include elasticity that is normally smooth, and quick water absorption and swelling properties that will allow the granules to be easily swallowed. We prepared the targeted granules by selecting GEL and SUC-GEL and CAR for matrix polymers that can gel at room temperatures or at cold temperatures, and we also included some additives.

Polymer gels used here were physical gels that

formed a three-dimensional unstructured network, and basic dynamic gel properties were known to be different at different gel concentrations and with different additives (*16,17*). We evaluated the additive effects of saccharides and found that granules with a high degree of elasticity can be prepared by adding XYL, SOR and MAL regardless of which polymer is used. However, a combination of CAR and MAL showed a decrease in elasticity compared with the other compositions. Granule elasticity seems to be affected by varying water-retaining properties and these unstructured networks showed elastic properties when XYL or SOR were added, which have a relatively high ability to retain water (*18*). Water absorption behavior was different depending on the type of polymer. GEL and SUC-GEL showed rapid water absorption behavior and variable differences with different saccharides. However, SUC-GEL granules tend to have a faster absorption speed and a larger amount can be absorbed compared with GEL. A combination of SUC-GEL with XYL had the fastest absorption. On the other hand, granules with CAR absorbed a large amount of water but the initial water absorption was slow regardless of the type of saccharide. This shows that CAR was not suitable to prepare the target granules. All granules showed a weight increase, and the polymer might swell and gel because of water absorption. Swelling of polymers generally occurs because a solvent enters and diffuses into the gel. This phenomenon of increasing the cubic volume by absorbing a solvent into a three-dimensional unstructured network made of a polymer compound occurs by placing it in a solution and increasing the crosslink density that causes a decrease in diffusion of the unstructured network and the degree of swelling (*19-21*). The affinity and osmotic pressure are responsible for this infiltration (*22,23*). Therefore, interaction between the unstructured network and the polymer, and the additives, affinity for water and osmotic pressure all affect the water absorption behavior. This happens with AA dissolution behavior. Granules with CAR showed slow dissolution behavior compared with granules with GEL and SUC-GEL, which showed rapid dissolution behavior. Granules with SUC-GEL tend to have a slightly higher initial dissolution ratio than granules with GEL, and this tendency was related to SUC-GEL rapid water absorption behavior.

From these results, it was observed that our granules can be prepared with a matrix of SUC-GEL and XYL or MAL as additives. However, we evaluated the effect of mixing SUC-GEL with CAR to take advantage of CAR's characteristics such as the ability to absorb a large amount of water even though the initial absorption is slow. Adding XYL or MAL to these granules improves their elasticity. However, initial water absorption behavior did not improve because CAR characteristics seemed to be significantly affected. Dissolution behavior was also slow in granules with CAR alone. These results show that, although it was affected by saccharides, water absorption that was affected too much by CAR could destroy the element of SUC-GEL. We identified SUC-GEL alone as the best matrix for our granules.

Thus, we focused on PEG, which is used as a plasticizer or a solubilizing agent, and evaluated the effect of adding PEG to SUC-GEL to improve the granules. Granules with PEG added at various mixing ratios were compared with granules with XYL added, and granules that included XYL had better elasticity than granules that included low amounts of PEG. By increasing the amount of PEG, the elasticity approached that of granules with XYL. The difference seemed to be in the water retention properties for the amount of PEG that was added. On the other hand, granules that included PEG had better water absorption behavior than granules that included XYL regardless of the amount of PEG that was added, and the rate of apparent water absorption was approximately triple that of granules that included XYL. These results seem to be because of differences in cross-linking and osmotic pressure, which are factors in gelling and swelling. Dissolution behavior of AA was the same as that of granules that included XYL, independent of the amount added.

Strength and water absorption behavior results showed that adding PEG was useful for improving water absorption. However, a large amount was PEG needed to obtain elasticity. We then evaluated the addition of PEG to the granules along with XYL, which resulted in a better preparation for the granules. Consequently, SUC-GEL/PEG (1) that contained a small amount of PEG had improved elasticity and maintained its rapid water absorption behavior. It had no effect for dissolution behavior. These results suggest that it was useful in preparing the target granules by adding PEG and XYL to SUC-GEL.

We evaluated polymers as a matrix and additives for a preparation of targeted oral agents that have elasticity and that are normally smooth, they also had to absorb water quickly and have swelling properties to make swallowing easier. We found that SUC-GEL was suitable for a matrix and that XYL, MAL and SOR were useful for improving elasticity. PEG was best for rapid water absorption, which was an important element in gelation. Additionally, we prepared the best target granules by selecting SUC-GEL as the matrix and then adding a small amount of PEG and XYL in same amount as for SUC-GEL.

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Original Article

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Effect of rice variety on the physicochemical properties of the modified rice powders and their derived mucoadhesive gels

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Summary **In the present study; the glutinous Niaw Sanpatong (NSP) and Niaw Koko-6 (NKK), and the non-glutinous Jasmine (JM) and Saohai (SH) were chemically modified. The difference of these rice varieties on the physicochemical characteristics of the modified rice powders and the properties of the derived gels were evaluated. X-ray diffractometer was used for crystalline structure investigation of the rice powders and gels. A parallel plate rheometer was used to measure the rheological property of the gels. It was found that the non-glutinous varieties produced gels with higher mucoadhesive properties than the glutinous rice. Rheological behavior of JM and SH gels was pseudoplastic without yield value whereas that of NSP and NKK gels was plastic with the yield values of 1077.4 ± 185.9 and 536.1 ± 45.8 millipascals-second (mPas), respectively. These different properties are considered to be due to the amylose content in different rice variety. The results suggest that the non-glutinous rice varieties with high amylose content are the most suitable for preparing gels as local delivery systems *via* the mucosal membrane.**

Keywords: Rice variety, rice gel, biodegradable, mucoadhesive, amylose content

1. Introduction

Rice (*Oryza sativa* L.) is the principal staple food for half the world's population. Rice grain is an important raw material for rice starch and nutrients like lipids and proteins. Moreover, rice bran is a rich source of bioactive antioxidant compounds (*1*). Rice powder is usually produced by milling whole rice grains including rice bran. Therefore, biodegradable products derived from rice powder are composed of various nutrients with antioxidant components.

Local delivery of drug *via* buccal, nasal, or vaginal mucosal membrane is receiving increased attention as for avoidance of acid hydrolysis in the gastrointestinal tract and hepatic first-pass effects (*2*). Drug delivery *via* mucosal membrane can be used for local therapy (*3,4*). Among several kinds of trans-mucosal dosage forms, gel is one of the most preferable because of its excellent mucoadhesiveness, comfort, and easy dispersion throughout the mucosa. Traditional gel bases are made of synthetic polymers, thus causing serious environmental problems. Gel bases derived from rice powder are of interest because of the advantages of rice mentioned above. However, rice varieties might affect the characteristics of the derived gels. Previous studies reported that there are great variations in the amylose content in the rice starch of different varieties (*5*). The amylose variation was reported to influence the properties of the films derived from rice (*6,7*). Therefore, it is necessary to study the relevant properties of various rice varieties to choose the one most suitable for formulation of appropriated gel bases as mucosal delivery systems. To our knowledge, there have been no previous studies examining the physicochemical characteristics of rice varieties which may affect the properties of the derived gels particularly on their rheological and mucoadhesive properties. The aim of this study was to investigate the effect of rice variety on the physicochemical properties of the modified rice powders and their derived mucoadhesive gels.

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2. Materials and Methods

2.1. *Rice materials and chemicals*

Milled rice grains of four common varieties in Thailand; Jasmine (JM), Saohai (SH), Niaw Sanpatong (NSP) and Niaw Koko-6 (NKK) harvested during April – September 2013 were used. These rice varieties are the most popular rice in Southeast Asian countries, particularly in Thailand. JM is classified as aromatic non-glutinous rice with pleasant odor. SH, NSP and NKK are the odorless rice varieties. NSP and NKK are classified as glutinous whereas SH is non-glutinous rice. Silver nitrate and monochloroacetic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and glacial acetic acid were from RCI Lab-scan Co., Ltd. (Bangkok, Thailand). All other chemicals and solvents were of AR grade or the highest grade available.

2.2. *Composition analysis of rice*

The composition analysis of the raw rice powders was performed according to the AOAC guidance (*8*). Kjeldahl nitrogen method was used for determining crude protein content with the nitrogen-to-protein conversion factor of 6.25. Soxhlet extraction method was used for fat content determination with petroleum ether as a solvent using a BUCHI Soxlet fat extraction unit over a 2 h period. Dry incineration in a muffle furnace at 550°C for 24 h was used to determine the ash content. Carbohydrates content was approximately determined by subtraction from the contents of the others components.

2.3. *Determination of amylose and moisture content*

Raw rice powder of each variety prepared by wet milling method previously described by Okonogi *et al*. (*7*), were analyzed for amylose content according to the method described by Juliano (*9*). Moisture content of the raw rice powder was determined using a Kett F-IA moisture content balance with halogen heating. The heating temperature was set at 105°C. The exact weight of sample before and after being heated to a constant weight was recorded. The moisture content of the samples was calculated on a wet basis.

2.4. *Modification of rice*

Rice modification was done according to the previous method described by Okonogi *et al*. (*7*) with some modification. Briefly, the raw rice powder was subjected to etherification using methanol-water mixture as a medium. The reaction was carried out in a 500-mL three necked round-bottom flask, equipped with motor-driven stirrer. A 50% sodium hydroxide aqueous solution was firstly mixed with methanol at a weight ratio of 1:4. After adding raw rice powder, the mixture was stirred until homogenous, and then proper amount of monochloroacetic acid was added. The mixture was stirred at 60°C for 3 h. The solid granules obtained were collected. The solid phase was washed several times with 95% ethanol until the silver nitrate test for chloride of the filtrate was negative. The dried solid product was pulverized. The modified rice powder that passed 80-mesh sieve was used for further studies.

2.5. *Morphology and internal solid structure of rice powders*

The morphology of rice particles were investigated by scanning electron microscope (SEM) using a JEOL JSM-5410LV (Japan) equipped with a large field detector. The acceleration voltage was 10-20 kV under low vacuum mode (0.7-0.8 torr). The internal solid structure of the obtained rice powders was characterized by means of X-ray diffraction (XRD) using a Siemens D-500 X-ray diffractometer with Cu Kα radiation at a voltage of 30 kV and 15 mA. The rice samples were scanned between $2\theta = 5\text{-}60°$ with a scanning speed of 5°/min. Prior to testing, the rice samples were dried at 50°C for 24 h and stored in a desiccator.

2.6. *Solubility index*

The solubility index of the rice powders was evaluated using the method previously described (*10*) with some modification. Rice powders (1 g) were gradually dispersed at room temperature in 250 mL of water by stirring at 100 rpm. After 1 h the solutions were filtered through Whatman (No. 1) filter papers and the weighed portions of the filtrate were dried at 60°C under vacuum until a constant weight was reached. The solubilization index was expressed as the percentage ratios of the solubilized rice/initial weighed rice. The higher of the index value, the higher the aqueous solubility of the samples.

2.7. *Gel preparation*

The modified rice powders were weighed and dispersed in distilled water to obtain 10% w/w rice dispersion. The dispersions were heated to 90°C in a closed chamber for 2 h and gently stirred to obtain homogenous gels without air bubble formation. The physical appearance of the gels was observed visually.

2.8. *Internal solid structure of gels*

The internal solid structure of the gels was investigated by using a Siemens D-500 X-ray diffractometer according to the procedures described in 2.4.1. The freshly prepared gel sample was studied without any prior treatment.

2.9. *Rheological behavior of gels*

The gels were investigated for their rheological behavior using a Brookfield rheometer R/S-CPS (USA) with a parallel plate and plate gap of 1000 µm. The gel sample was gently loaded onto the rheometer plate using a microspatula. Care was taken to minimize shearing during sample removal and sample loading. The rheological behavior of the samples was characterized over a range of 0-1000 s^{-1} for a period of 3 min. All studies were done at 30 ± 2°C. The measurements were made in triplicate. The stress-shearing rate profile of any gel was obtained from a plot of shear stress versus shear rate under the given experimental conditions. The rheological parameters of the samples such as average apparent viscosity and yield value were calculated by using Rheo3000 program.

2.10. *Mucoadhesive study*

The gels were examined for mucoadhesive property using an *ex vivo* mucoadhesive test previously described (*11*) with some modification. Gels with the exact weight of 1 g were applied homogenously on a 2 cm × 2 cm area of a porcine intestinal mucosal membrane. After that the membrane was fixed on the internal side of a beaker which was kept in a bath at 37 ± 1°C. The beaker was filled with 800 mL water at the same temperature. A 150 rpm stirring rate was subsequently applied to simulate the *in vivo* cavity environment to which the gels will be subjected. The time of gel detachment or disappearance from the membrane was recorded.

2.11. *Statistical analysis*

Descriptive statistics for continuous variables were calculated and reported as a mean ± standard deviation. Data were analyzed using a one-way analysis of variance and Duncan's multiple range test ($p < 0.05$) using SPSS software version 11.

3. Results and Discussion

3.1. *Composition analysis*

The content of chemical compounds defines the nutritional quality of rice. It is well known that the major nutritional component of rice grain is carbohydrate at approximately 70-90% or more. This difference is dependent on many factors including the variety of rice. It was previously reported that total carbohydrate content in the brown rice grains of six varieties grown in the Philippines was between 72-82% (*5*). Starches existing in different rice varieties differ in stability. Sagum and Arcot (*12*) reported that three raw rice varieties had similar total carbohydrate content of 82-83% w/w. The four native rice varieties used in the present study contained carbohydrate content of approximately 91-92% (Table 1), significantly higher than that previously reported. The non-glutinous rice showed slightly higher carbohydrate content than the glutinous varieties. The glutinous varieties had slightly higher protein and lipid but less ash than the non-glutinous rice. Our results of protein content of the glutinous rice was consistent with Keeratipibul *et al*. (*13*), who worked with similar Thai glutinous rice varieties and reported their protein contents of 7.0-7.5%. However, Thumrongchote *et al.* (*14*), who worked with different non-glutinous varieties, reported their protein content slightly less than our results. This was considered to be due to the difference in rice varieties. Fat content for all the rice varieties evaluated ranged between 0.4-0.7% which is in good agreement with other previous reports, while ash content of our white rice samples ranged between 0.2-0.4% which significantly lower than that of the black rice varieties reported previously (*15*).

Table 1. Composition of the raw rice powders

Composition (%)	Rice Varieties			
	Non-glutinous rice		Glutinous rice	
	JM	SH	NSP	NKK
Carbohydrates	92.0 ± 0.8	92.1 ± 0.2	91.5 ± 0.8	91.9 ± 0.3
Lipids	0.5 ± 0.3	0.4 ± 0.3	0.7 ± 0.3	0.6 ± 0.1
Proteins	7.2 ± 0.4	7.2 ± 0.1	7.4 ± 0.2	7.3 ± 0.5
Ash	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1

Moisture content and moisture migration can cause physical, chemical and biological changes in materials (*16*). According to the definition, moisture content is expressed on a wet basis or a dry basis. In this study, the moisture content of the rice samples was calculated as the wet basis. Among the four rice varieties, NKK showed the lowest moisture content of 2.6% whereas SH showed the highest value of 7.2%. The moisture contents of JM and NSP were similarly moderate level at 3.4 and 4.3%, respectively. Moisture content of raw rice influences its storage properties as it is related to the water activity of microorganisms (*17*). The results in the present study suggest that attention should be paid to the storage of rice with high moisture content such as SH because of the high risk of microorganism contamination.

The amylose content among the four rice varieties was significantly different. SH had the highest amylose content of 21.8 ± 0.3 % followed closely by JM (17.5 ± 0.5%) whereas NSP presented the lowest amount of 4.0 ± 0.5% followed by NKK (7.4 ± 0.4%). It was noted that the amylose content in the non-glutinous rice was significantly higher than that of the glutinous rice. These quantity differences of rice compositions mutually influenced the characteristics of their respective modified rice powders and gels.

3.2. *Modification of rice*

Preferable hydrophilic gels can be formed by using gelling agents with high ability to dissolve in water.

However, raw rice starch has low water solubility. Therefore, modification of rice starch based on physical, chemical, and biological reactions has been suggested to solve this problem (*18*). In the present study, chemical modification based on carboxymethylated etherification was used because the modified starches obtained from this etherification reaction were reported to cover a wide range of applications including pharmaceutical and biomedical fields (*19*). The color of the obtained modified rice powders of the four varieties was off-white while that of the color of the original raw rice powders was pure white.

3.3. *Morphology of rice particles*

Particle morphology and approximate size of the raw rice powders in comparison with the modified rice powder under SEM analysis is demonstrated in Figure 1A. The raw rice particles of both glutinous and non-glutinous varieties displayed an irregular polygonal shape with several obvious sharp edge surfaces. Our findings on the shape of rice powders are similar to those previously reported (*20*), but the size of the rice particles was not in agreement. This may be due to the difference in rice varieties and the method of preparation of rice powders. A layered organization was clearly observed in NSP and some parts of JM particles. Small pieces, possibly "broken particles" produced by the preparation process were observed attached to the surface of the large particles (*21*). Small particles were more commonly observed in the glutinous than in the non-glutinous rice. Among the four rice varieties, JM particles showed the smallest size granules of about 3-6 μm whereas the average size of the others was approximately 5-10 μm. In comparison with the raw rice, the chemical modification under the present conditions caused a significant change to the rice particles as seen in Figure 1B. The modified JM particles were slightly swollen and the surface edges were blunt. The particles size was slightly larger (5-10 μm) than the raw rice (3-6 μm). The change in SH particles after modification was similar to that of JM in terms of particle swelling and edge blunting. However, modified SH particles also exhibited a high degree of alteration in terms of particle clustering or merging. Interestingly, the modification produced noticeable changes in glutinous rice varieties. The modified rice particles of both NSP and NKK displayed prominent changes in shape and surface with extremely higher swelling and merging than the non-glutinous rice, in that some particles of the rice appeared to be completely fused and that individual particles could not be observed. The surface of the modified NSP and NKK rice appeared rough and wrinkled. Uneven structure with fine porosity was clearly observed. It was proposed that the alkaline environment during the carboxymethylation reaction accounts for the structural changes. A previous report indicated that amylopectin is responsible for the swelling

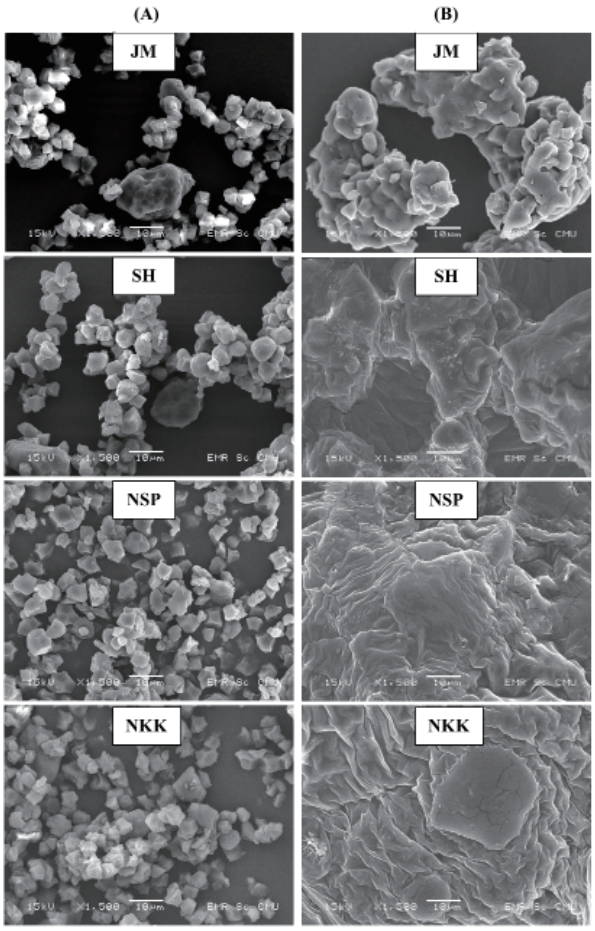

Figure 1. SEM micrographs of the raw (A) and modified (B) rice powders.

property of starch, whereas amylose acts as a connector to hold the starch particles intact (*22*). However, recent research reported that the extent of rice swelling after modification was due to the amylose content (*20*). In the present study, the glutinous rice which contained significantly less amylose demonstrated higher swelling and microstructural change than the higher amylose content, non-glutinous varieties like JM or SH. We propose that the results of the present study are due to the mutual activity of amylose and amylopectin content as well as the other components existing in the rice powders after modification.

3.4. *Internal solid structure of rice particles*

The XRD is used to reveal the presence and characteristics of internal crystalline structures of starch particles. Crystalline starch lattices are formed due to the arrangement of amylose and amylopectin molecules which can be classified to A-, B- and C-type by XRD pattern analysis (*23*). A-type starch has strong diffraction peaks at about 15° and 23° with unresolved doublet at around 17° and 18°. B-type starch gives a characteristic peak at about 6° and the strongest diffraction peak at

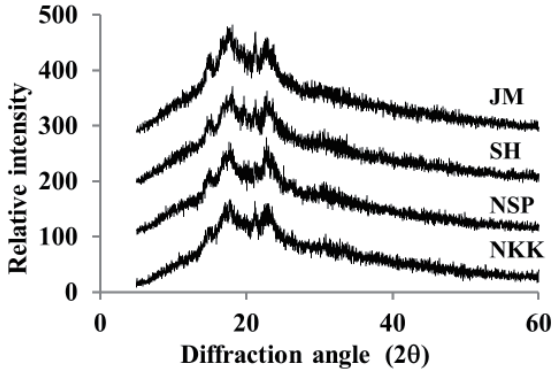

Figure 2. XRD patterns of the raw rice powders.

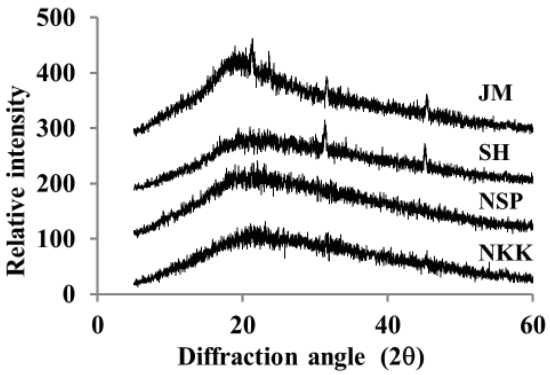

Figure 3. XRD patterns of the modified rice powders.

Table 2. XRD diffraction angles of the raw rice and solubility index of the rice powders

Rice variety	XRD diffraction angles (2θ)	Solubility index (%)	
		Raw rice	Modified rice
JM	14.9, 17.2, 20.5, 22.9	0.2 ± 0.1	97.6 ± 0.1
SH	14.8, 17.2, 20.5, 22.8	0.3 ± 0.1	99.8 ± 0.2
NSP	14.7, 17.1, 22.7	0.0 ± 0.0	93.1 ± 0.2
NKK	14.5, 16.9, 21.0, 22.7	0.0 ± 0.0	94.9 ± 0.1

around 17° with some small peaks at about 15°, 20°, 22°, and 24°. B-type starch is more resistant to enzyme hydrolysis than A-type starch (*24*). C-type starch is a mixture of A and B-type polymorphs and shows strong diffraction peaks at about 6° and 15° (according to B-type) and 17° and 23° (according to A-type). The C-type can be further classified as CA-type (closer to A-type) and CB-type (closer to B-type) according to the proportion of A- and B-type polymorphs. The XRD patterns of the raw rice samples of the four tested varieties are shown in Figure 2. It was found that all raw rice samples displayed strong reflections with different diffraction angles (Table 2). The diffraction peaks around 14.5-14.9°, 16.9-17.2° and 22.7-22.9° were considered to be the A-type crystalline arrangement. The minor crystalline peaks similar to B-type was observed in the XRD patterns of JM, SH and NKK around 20.5-21.0°. Starches from cereals usually present A-type structure. However, only NSP showed the characteristics of A-type structure. The crystalline structure of JM, SH and NKK were considered as CB-type because they showed one peak that seemed to be a typical of B-type. The different results of JM, SH and NKK from the previous reports on the type of crystallinity may have been largely due to differences in amylose content in the tested rice varieties, and to a lesser extent an effect of the process of rice powder preparation prior to XRD measurement.

After modification, the internal solid structure of the rice obviously changed. XRD halo patterns were found indicating the change of crystalline structure to an amorphous form. However, the level of crystalline destruction was different among the four rice varieties. As seen in Figure 3, the internal structure of the two glutinous rice varieties were completely changed to an amorphous structure whereas some crystalline peaks were still observed in the non-glutinous varieties. All the original XRD crystalline peaks of SH disappeared after modification, and were replaced by new crystalline peaks at the diffraction angles of 31.2° and 45.0°. The XRD of the modified JM was similar to that of the modified SH as far as the new crystalline peaks, but one small original crystalline peak at 20.5° still remained. The XRD results were in agreement with the morphological changes observed in the SEM. The results suggested that the carboxymethylation conditions used in the present study caused the complete loss of crystallinity in glutinous rice varieties but not totally for the non-glutinous rice. It appears that the crystalline solid structure of the low amylose rice powders is easily destroyed by chemical modification. Some small peaks remaining in the XRD patterns of the modified rice of the high amylose varieties suggest the less destruction of amylose double helices, and that the new crystalline structure might be formed after chemical modification. These results suggest that the non-glutinous varieties need higher different conditions for chemical modification than the glutinous varieties.

3.5. *Solubility index*

Water miscibility is important for gelling substances in order to form desirable hydrophilic gels. Gelling agents with high water solubility can provide hydrophilic gels with the preferable characteristic of high transparency. In the present study, rice powders of the four varieties demonstrated significantly different solubility properties as shown in Table 2. It was also clearly seen that the modified rice powders have extremely higher aqueous solubility than the raw rice. This result confirmed that substitution of –OH with $–CH_2–COO–$ group during rice modification enhances its water solubility. In addition, it is known that the granules disruption during modification highly increased the hydrophilic character of the starch. This effect also enhances the water solubility of the modified starches. The results demonstrated that the

non-glutinous varieties had significantly higher water solubility than the glutinous rice. It is also noted that between the two non-glutinous rice varieties, SH was higher soluble than JM. With respect to the glutinous varieties, NKK, whose amylose content is higher, showed higher water solubility than NSP. These results indicate that the solubility of modified rice powders is directly related to amylose level. These findings were in good agreement with those previously reported (*25*).

3.6. *Appearance and internal solid structure of the gels*

It is known that rice gels prepared by thermal pregelatinization gradually retrograde during storage. To retard or inhibit the retrogradation of rice starch molecules, chemical modification has been introduced to starch granules (*26*). In the present study, the four rice powders were chemically modified by etherification prior to gel preparation. All of the prepared gel formulations were similar in outward appearance as transparent semisolids and had a homogeneously desirable external structure. The non-glutinous gels were colorless whereas some color was observed in the glutinous NSP and NKK rice gels. The color of NSP gels was pale yellow while that of NKK gels was pale fulvous. The result of the internal crystalline structure analysis of the gels by XRD is presented in Figure 4. The XRD diffraction of all rice gels exhibited the complete halo patterns indicating that the crystalline structure of all modified rice particles was completely destroyed. It is noted that the crystalline structure of the modified non-glutinous powders disappeared when the rice powders were transformed into a gel structure. This result indicates that the condition of gel preparation as well as the concentration of rice powders in the gels was suitable and that the water quantity was sufficient for complete hydration of the rice particles to form the non-crystalline network structure of the gels.

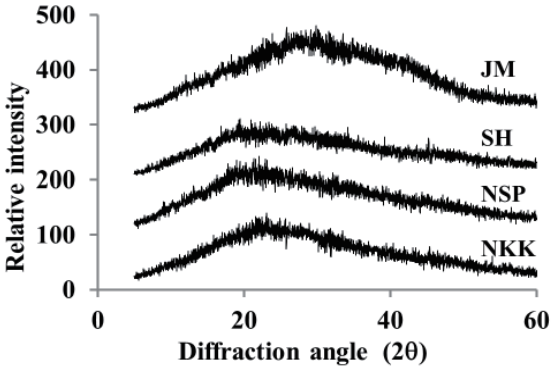

Figure 4. XRD patterns of the modified rice gels.

3.7. *Rheological behavior of the gels*

Rheological property is very important for pharmaceutical gel formulations because this knowledge can provide the microstructural environment or flow behavior information of the gel which is responsible for drug diffusion and compatibility. The rheological behavior of the obtained rice gels is shown in Figure 5. The stress-strain relationship was not linear for all

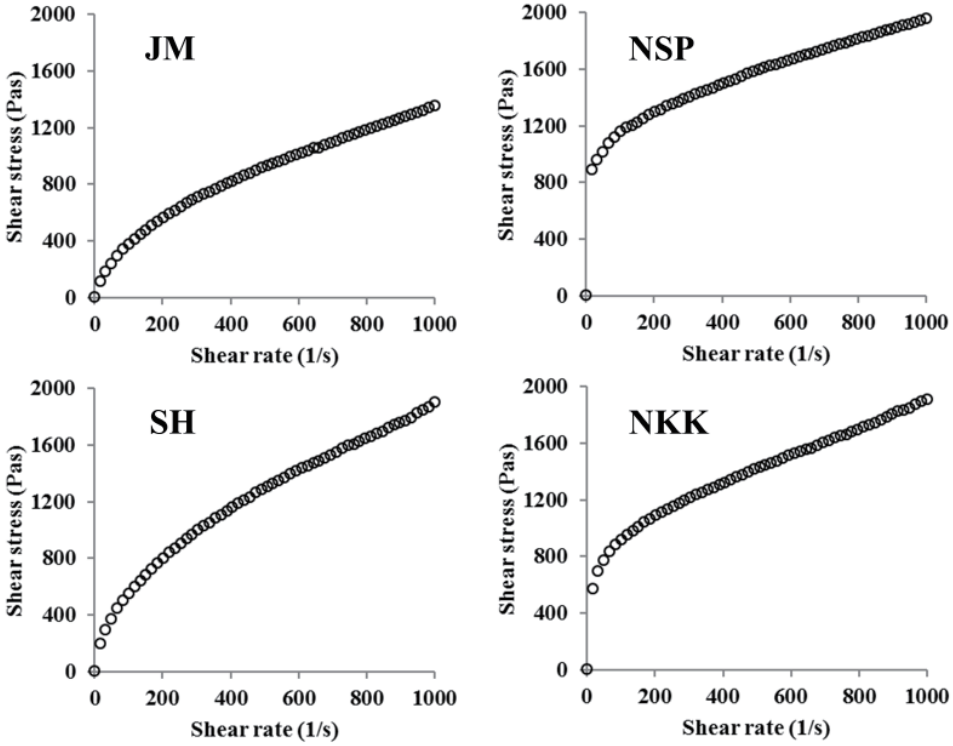

Figure 5. Rheograms of the modified rice gels.

Table 3. Rheological parameters and mucoadhesive properties of the modified rice gels

Rice variety	Rheological parameters		Mucoadhesion
	Viscosity (mPas)	Yield value (mPas)	Detachment time (min)
JM	2192.9 ± 54.7	→ 0	45 ± 2
SH	3167.4 ± 37.4	→ 0	56 ± 4
NSP	6213.1 ± 540.9	1077.4 ± 185.9	30 ± 3
NKK	4392.9 ± 315.3	536.1 ± 45.8	35 ± 2

samples suggesting that the rheological property of the obtained rice gels was a non-Newtonian flow and the gel viscosity was changed when the gels were sheared. This result indicates the incomplete formation of gel structure because of the lower shear stress. The non-glutinous JM and SH gels showed pseudoplastic flow indicating an immediate flow after stress application whereas the glutinous NKK and NSP gels showed plastic flow with yield value. Yield value is defined as the minimum stress that must be applied before the material really starts to flow (*27*). In pharmaceutical fields, it is very important to know the viscosity and yield value of the gel formulations because many passages (tubes, buccal applicators) are required for their packaging and administration. The viscosity and the yield value of the formulated gels are shown in Table 3. The viscosity of the glutinous rice gels was found to be significantly higher than that of the non-glutinous gels. Among the four varieties, the gel of NSP presented the highest viscosity of about 6 pascals-second (Pas) whereas that of JM displayed the lowest viscosity of approximately 2 Pas. Gels with yield value present advantage that the gels that do not flow out of their container due to their own weight if the container is inverted. However, the yield value of the developed glutinous gels is too high so as to offer significant resistance during application on the applied surfaces. Between the two glutinous varieties, the yield value of the NSP gel was significantly higher than that of NKK gel. Previous research reported that the rheological properties of the rice gels developed by thermal gelatinization were influenced by amylose content and concentration of starch (*28*). In the present study, the rice concentration in the gel formulations was fixed at 10%. Therefore, the variation of rheological behavior of the rice gels was considered to be influenced by mutual interaction of rice components particularly amylose content and water in the gels.

3.8. *Mucoadhesive property of the gels*

Mucoadhesive property is important in pharmaceutical formulations for trans-mucosal application in both localized and systemic drug delivery system. It was reported that the enhancement of drug delivery through mucosal membranes could be successfully achieved by ensuring the sufficient mucoadhesive strength of the delivery systems (*29*). Gels with high mucoadhesive properties can be retained in the application area for the desired duration of action whereas those without this property are easily washed away and removed by the surrounding medium such as saliva, nasal or vaginal fluids in buccal, nasal, and vaginal cavities, respectively. Therefore, mucoadhesive property is an important issue for prepared gels administered *via* mucosal membranes. Table 2 illustrates the time required for gel detachment from the mucosal membrane after immersed in the water at body temperature with a stirring speed of 150 rpm. As can be seen, the non-glutinous rice gels showed significantly longer adhesion times indicating higher mucoadhesive strength than the glutinous rice gels. Moreover, it was observed that the non-glutinous rice gels were gradually dissolved in the medium until exhausted while the glutinous rice gels were ruptured by the stirring force and some small pieces were detached from the gels. Mucoadhesion is the interfacial force between the drug delivery system and the mucus layer coating an epithelium. Basic theories such as adsorption, wetting and diffusion phenomena have been described and associated with the mechanisms by which mucoadhesion occurs (*30,31*). These phenomena are enhanced by substances having more hydrophilic properties. As higher lipid content was found in the glutinous than in the non-glutinous varieties, it is thus considered to be a cause of lower adsorption force and wetting property as well as surface energy interactions of the glutinous gels when spreading onto the membrane.

4. Conclusion

The effects of rice varieties on the physicochemical characteristics of rice powder and properties of the derived gels obtained from the respective modified rice powders were explored in this study. The glutinous NSP and NKK and the non-glutinous JM and SH rice varieties yielded carboxymethylated modified rice powders with different morphology, crystallinity and aqueous solubility characteristics. Higher amylose content in the non-glutinous rice varieties significantly affected the internal crystalline structure of the rice powders and mucoadhesive as well as rheological properties of the respective derived gels. Rheological behavior of the non-glutinous JM and SH gels was pseudoplastic flow without yield stress whereas the glutinous NSP and NKK gels were more viscous and exhibited plastic flow with obvious yield values. These different gel properties reflect the different rice varieties used for gel preparation. The results suggest that non-glutinous rice varieties with high amylose contents are the most suitable for preparing pharmaceutical gels for trans-mucosal systems.

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Original Article

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Generic Selection Criteria for Safety and Patient Benefit [IV] − Physicochemical and pharmaceutical properties of brand-name and generic ketoprofen tapes

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Summary **The physicochemical and pharmaceutical properties (pH, peel force, water-vapor permeability, and stretchability) of brand-name and generic ketoprofen products were evaluated and compared. The pHs of Mohrus as a brand-name product and Teikoku as a generic product were low (about 4). Among the other generic products, Patell and Nichi-Iko had a pH of about 4.3 while Frestol, Raynanon, BMD, and Touchron showed a pH of 4.6-5.2, which was in the pH range of normal healthy skin (4.5-6.5). The adhesive force was high (≥ 1.38) for Mohrus as a brand-name product as well as for Teikoku and Patell as generic products, but it was low (≤ 0.57) for the other 5 generic products. The water-vapor permeabilities of Mohrus as a brand-name product and Teikoku and Patell as generic products were low, being less than 1/6 of those for the other 5 generic products. Among the 5 generic products, BMD showed the highest water-vapor permeability (1,330 g/m^2), and the other products also showed a value ≥ 1,100 g/m^2. The elongatedness of Mohrus was the lowest (15.5 cm), and that of Raynanon was the highest (24.5 cm); the difference was 9 cm. In this study, the physiochemical and pharmaceutical properties of ketoprofen tapes were clarified, which will allow pharmacists to provide products according to the needs of each patient when a brand-name product is changed to a generic one.**

Keywords: Ketoprofen tape, generic, abrasion power, water-vapor permeability, pharmaceutical properties

1. Introduction

In Japan, several generic drugs are commercially available to replace brand-name drugs. As health expenditure for the elderly will markedly increase in the future, the widespread use of generic drugs whose prices are low is being promoted (*1*). In clinical practice, whether or not generic drugs should be selected is evaluated by insurance pharmacists, excluding prescriptions for which there is a check in the column of "impossible to change" and an insurance doctor's signature is present (*2*). When selecting generic drugs, information on differences in the efficacy and quality between brand-name and generic products is necessary.

In the field of orthopedics, a non-steroidal anti-inflammatory analgesic agent, Ketoprofen tape, which is effective for low back pain, osteoarthrosis, muscle pain, and rheumatoid arthritis, has recently been routinely used in clinical practice (*3*). This tape is characterized by its adhesive force persisting for many hours, achieved even in articulating regions, such as the knees and elbows. However, when removing this tape, the corneal layer of the skin is exfoliated due to its high peel force (adhesive force), leading to pruritus, pain, or rubefaction. For this reason, a dosage form design to reduce skin irritation is required (*3,4*). Currently, many generic products of Ketoprofen tape are commercially available from various pharmaceutical manufacturers. The base component other than the active ingredient, support material, and manufacturing process are based on each manufacturer's individual techniques.

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Pharmacists must select products meeting patients' wishes, considering the properties of each product, and explain about them to patients. In particular, it is necessary to select preparations appropriate for patients from many generic drugs. In addition, some products are commercially available not only by prescription but also as over-the-counter (OTC) drugs. Pharmacists' knowledge and evaluation on selecting products are important. However, few studies have reported the properties of various preparations, which may become selection criteria. They are not sufficiently utilized in clinical practice (*5,6*). In this study, we evaluated the physicochemical properties of brand-name (*7*) and generic products of Ketoprofen tape, and examined criteria for selecting these products as information useful in clinical practice.

2. Materials and Methods

2.1. *Materials*

As Ketoprofen tape, a brand-name product, Mohrus® Tape L40 mg (Hisamitsu Pharmaceutical Co., Inc., Tokyo, Japan), and generic products, such as Ketoprofen tape 40 mg "Teikoku" (Teikoku Seiyaku Co., Ltd., Kagawa, Japan), Patell® tape 40 (Oishi Koseido Co., Ltd., Saga, Japan), Frestol® tape 40 mg (Towa Pharmaceutical Co., Inc., Osaka, Japan), Raynanon® tape 40 mg (Shiono Chemical Co., Ltd., Tokyo, Japan), Ketoprofen tape 40 mg "BMD" (Biomedix Co., Ltd., Tokyo, Japan), Touchron® tape 40 (Kyukyu Pharmaceutical Co., Ltd., Tokyo, Japan), and Ketoprofen tape 40 mg "Nichi-Iko" (Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan), were purchased, and used in this experiment (Table 1). All the other reagents were of analytical grade.

2.2. *Measurement of pH*

We measured pH values, as described by Ohtani *et al*. (*5*). Briefly, each preparation was cut into sections measuring 70 × 50 mm, placed in sample bottles containing 20 mL of purified water, and agitated for 24 h. Subsequently, the pH of the solution was measured using a Benchtop pH meter F-74 (HORIBA, Ltd., Kyoto, Japan). For each product, measurement was conducted 3 times, and the mean was adopted as its pH value.

2.3. *Measurement of the peel force*

The peel force (adhesive force) was measured according to the adhesive tape/sheet test method established in the Japanese Industrial Standards (JIS) (*8*) and method described by Miura *et al*. (*9*). Briefly, a laboratory jack was fixed on an experimental table, and the unilateral side of the jack was rolled with Controlled Caliper Ethylene Vinyl Acetate membrane (EVA, 3M CoTran™ 9702) as a type of artificial skin. On its surface, a section of each product measuring 30 × 52 mm was longitudinally attached. In addition, a cylindrical weight (4 kg) was rolled over each section to a specific site and back. Each section was allowed to stand for 30 minutes. Subsequently, the peel force was measured by pinching a 2-mm area of the upper margin with a clip and pulling it at a constant rate (1 mm/sec) so that the adhesive surface was vertical to a digital force gauge, ZTS-20N (Imada Co., Ltd., Aichi, Japan), until the tape had been completely exfoliated from the EVA membrane. With respect to each product, measurement was conducted 3 times, and the mean was regarded as the peel force.

2.4. *Measurement of the water-vapor permeability*

A water-vapor permeability test was performed, as described by Hiyoshi *et al*. (*3*). Briefly, 10 mL of purified water was placed in a glass container, and its opening was covered with a round section of each product measuring 40 mm in diameter. After the periphery was fixed with a piece of elastic paraffin film (Parafilm: Pechiney Plastic Packaging Company, U.S.A.), the weight was measured. Subsequently, each sample was allowed to stand for 24 h under the following conditions: temperature, 40°C; relative humidity, 50%. Additionally, the weight was measured. The water-vapor permeability was calculated from the rate of change in the weight using the following formula: Water vapor permeability ($g/m^2 \cdot 24$ h) = $(W_0 - W_1) \cdot 10{,}000/A$ [W_0: Weight before testing (g), W_1: Weight after testing (g), A: Area of the glass container's opening (cm^2)]. With respect to each product, measurement was conducted 3 times, and the mean was regarded as the water-vapor permeability.

Table 1. Various products have been used in this experiment

Product name	Class	Abbreviated name	Company	Serial number
Mohrus® Tape L40 mg	brand-name	Mohrus	Hisamitsu Pharmaceutical Co., Ltd.	LC12U
Ketoprofen tape 40 mg "Teikoku"	generic	Teikoku	Teikoku Seiyaku Co., Ltd.	7104
Patell® tape 40	generic	Patell	Oishi Koseido Co., Ltd.	621110
Frestol® tape 40 mg	generic	Frestol	Towa Pharmaceutical Co., Ltd.	A007
Raynanon® tape 40 mg	generic	Raynanon	Shiono Chemical Co., Ltd.	ZS03
Ketoprofen tape 40 mg "BMD"	generic	BMD	Biomedix Co., Ltd.	1W17
Touchron® tape 40	generic	Touchron	Kyukyu Pharmaceutical Co., Ltd.	1Y11
Ketoprofen tape 40 mg "Nichi-Iko"	generic	Nichi-Iko	Nichi-Iko Pharmaceutical Co., Ltd.	1S12

2.5. *Measurement of the stretchability*

The end (10 mm) of a section of each product measuring 20 × 100 mm was fixed on an experimental table with the adhesive surface facing upward. The maximum extension distance (cm) was measured by pulling the diagonal side. For each product, measurement was conducted 6 times, and the mean was regarded as the stretchability.

2.6. *Statistical analysis*

The values were compared using *Welch's t*-test. A *p*-value of 0.05 or 0.01 was regarded as significant.

3. Results

3.1. *Measurement of pH*

The results of pH measurement of each product are shown in Figure 1. Some brand-name and generic products showed differences in the pH. In particular, the pH values of the brand-name product, Mohrus, and a generic product, Teikoku, were 4.0 and 3.9, respectively. On the other hand, concerning the other generic products, the pH values of Patell and Nichi-Iko were 4.3 and 4.4, respectively. In addition, they were comparable to those of Frestol (pH 4.8), Raynanon (pH 4.6), BMD (pH 5.2), and Touchron (pH 4.8). Furthermore, significance tests were conducted to compare brand-name products with various generic products. Frestol and BMD showed significant differences in comparison with the brand-name product, Mohrus ($p < 0.01$) (Figure 1).

3.2. *Measurement of the peel force*

The peel force (adhesive force) of each product was measured. The results are presented in Figure 2. The peel forces of the brand-name product, Mohrus (1.38 N), and generic products, Teikoku (1.82 N) and Patell (1.77 N), were high, but those of the other generic products, Frestol (0.43 N), Raynanon (0.57 N), BMD (0.51 N), Touchron (0.45 N), and Nichi-Iko (0.50 N), were low. In addition, significance was tested between the brand-name and generic products. There were significant differences in the peel force between Mohrus and Frestol/Touchron/Nichi-Iko/BMD/Raynanon ($p < 0.01$)/ Teikoku ($p < 0.05$) as shown in Figure 2.

3.3. *Measurement of the water-vapor permeability*

The water-vapor permeability of each product was measured. The results are shown in Figure 3. We compared the water-vapor permeability of each product. There were marked differences among the products. Briefly, the water-vapor permeabilities of the brand-name product, Mohrus (117 g/m^2), and generic products, Teikoku (95 g/m^2) and Patell (85 g/m^2), were low, whereas those of the other generic products, Frestol (1,125 g/m^2), Raynanon (1,200 g/m^2), BMD (1,338 g/m^2), Touchron (1,125 g/m^2), and Nichi-Iko (1,231 g/m^2), were high. In addition, significance was tested between the brand-name and generic products. There were significant differences in the water-vapor permeability between Mohrus and Touchron/Frestol/Raynanon/Nichi-Iko/BMD ($p < 0.01$) as shown in Figure 3.

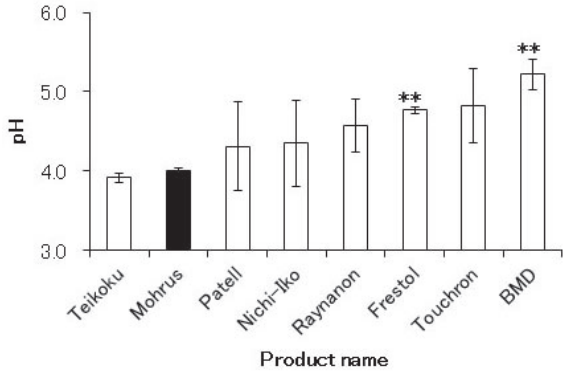

Figure 1. pH comparison of various products (n = 3). ■, brand-name product; □, generic product; $^{**}p < 0.01$ (brand-name *vs*. various generics; *Welch's t*-test)

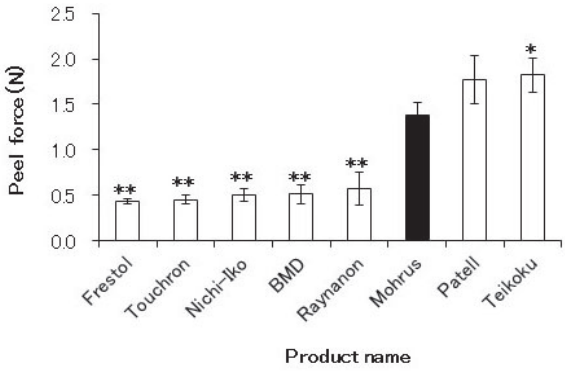

Figure 2. Comparison of the peel force (N) of various products (n = 3). ■, brand-name product; □, generic product; $^{*}p < 0.05$, $^{**}p < 0.01$ (brand-name *vs*. various generics; *Welch's t*-test)

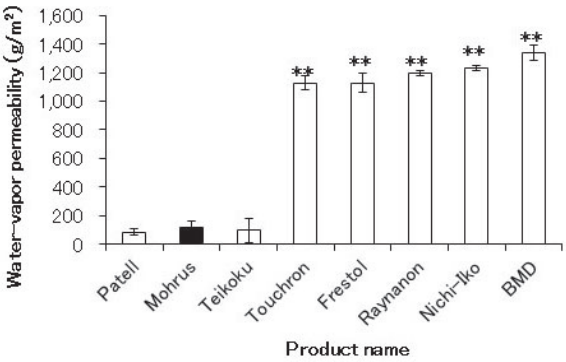

Figure 3. Comparison of the water-vapor permeability (g/m^2) of various products (n = 3). ■, brand-name product; □, generic product; $^{**}p < 0.01$ (brand-name *vs*. various generics; *Welch's t*-test)

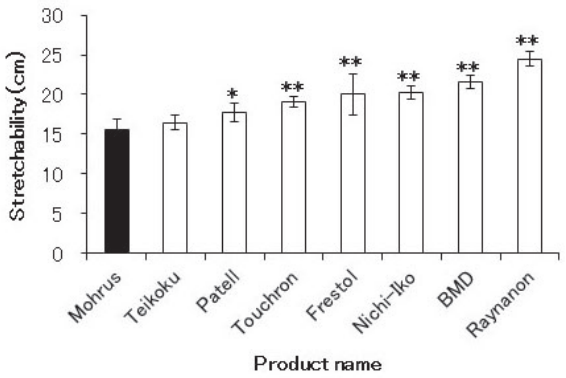

Figure 4. Comparison of the stretchability (cm) of various products (n = 6). ■, brand-name product; □, generic product; $^{*}p < 0.05$, $^{**}p < 0.01$ (brand-name *vs*. various generics; *Welch's t*-test)

3.4. *Measurement of the stretchability*

The stretchability of each product was measured. The results are presented in Figure 4. The stretchabilities of the brand-name product, Mohrus (15.5 cm), and a generic product, Teikoku (16.5 cm), were low. Those of the other generic products, Patell (17.7 cm), Touchron (19.1 cm), Frestol (20.0 cm), Nichi-Iko (20.2 cm), BMD (21.6 cm), and Raynanon (24.5 cm), were high. In addition, significance was tested between the brand-name and generic products. There were significant differences in the stretchability between Mohrus and Touchron/Frestol/Nichi-Iko/BMD/Raynanon ($p < 0.01$)/ Patell ($p < 0.05$) as shown in Figure 4.

4. Discussion

4.1. *Measurement of pH*

The pH of healthy skin ranges from 4.5 to 6.5 (slightly acidic). As tape preparations are directly attached to the skin, their pH may markedly influence patients' impressions of their use. The pH of each product is shown in and Figure 1. There were differences in the pH among the products. In particular, the pH values of the brand-name product, Mohrus, and a generic product, Teikoku, were approximately 4.0. Concerning the other generic products, the pH values of Patell and Nichi-Iko were approximately 4.3. In addition, those of Raynanon, Frestol, Touchron, and BMD ranged from 4.6 to 5.2, within a healthy skin pH range of 4.5 to 6.5. These results suggest that Mohrus and Teikoku become skin-stimulating factors, because their pH values were lower than the pH range of healthy skin.

4.2. *Measurement of the peel force*

The peel force (adhesive force) of tape preparations may directly influence impressions of their use, such as ease of peeling, in accordance with its intensity. Tape preparations with a weak peel force may come off due to frictions with clothes or in articulating regions, such as the elbows and knees. We measured the peel force (N) of each product. The results are presented in Figure 2. The peel forces of the brand-name product, Mohrus, and 2 generic products, Teikoku and Patell, were high (1.38 N or higher). However, those of the other generic products were low (0.57 N or lower). These results showed that there were differences in the peel force among the products, suggesting that impressions of their use markedly differ. These 3 tape preparations with a high peel force (Mohrus, Teikoku, and Patell) may not come off even in articulating regions, facilitating application for a relatively long period. However, at the time of exfoliation, they may cause detachment damage of the stratum corneum, in which the corneal layer of the skin surface is simultaneously peeled, and physical stimuli derived from dermal stress may lead to support stress, inducing symptoms such as hypersensitivity, erythema, eruption, rubefaction, swelling, feeling of stimulation, and pruritus (*7*).

4.3. *Measurement of the water-vapor permeability*

Tape preparation-related stimulations of the skin include physical stimuli on exfoliation, drug allergy, chemical stimuli related to additives/impurities, and maceration stimuli, such as corneal-layer hydration, sweat-gland blocking, and bacterial proliferation associated with occlusion at the site of attachment (*10*). Of these, maceration stimuli may occur in areas to which products with a low water-vapor permeability are attached. Therefore, we measured the water-vapor permeability of each product. The results are shown in Figure 3. We compared the water-vapor permeability of each product. There were marked differences among the products. Briefly, the water-vapor permeabilities of the brand-name product and 2 generic products (Teikoku and Patell) were low (1/6 or less of those of the other 5 generic products). On the other hand, of the 5 generic products, BMD showed the highest water-vapor permeability (1,338 g/m^2). The values of the other products were also 1,100 g/m^2 or higher. The products used in this experiment were designed to be applied over 24 h (attached once a day). Therefore, the use of products with a high permeability may prevent maceration stimuli (*11*). However, an increase in the permeability reduces the sealing property, decreasing skin transfer of the drug. Therefore, when selecting products, it may be important to sufficiently understand their properties (*4*).

4.4. *Measurement of the stretchability*

Ketoprofen tape preparations are attached to articulating regions, such as the lumbar spine, elbows, and knees, in many cases. Therefore, the stretchability of tape preparations is considered an important factor. In

particular, when tape preparations are attached to the elbows or knees, they may promptly come off due to unfavorable stretchability. The stretchability of each product is shown in Figure 4. There was a 9-cm difference between Mohrus (15.5 cm: minimum) and Raynanon (24.5 cm: maximum). The stretchability may be related to the material composition of each product. Low-stretchability products may restrict dermal expansion and contraction, stimulating the site of attachment with tension.

The results of this study showed that there were differences in physical properties among Ketoprofen tape preparations. This makes it possible for pharmacists to provide products based on patients' wishes (products that are painless on peeling them off, and those that are breathable) when switching a brand-name drug to a generic.

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