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Optimal cut-off values for the homeostasis model assessment of insulin resistance (HOMA-IR) and pre-diabetes screening: Developments in research and prospects for the future

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Summary **Diabetes mellitus (DM) appears to be increasing rapidly, threatening to reduce life expectancy for humans around the globe. The International Diabetes Federation (IDF) has estimated that there will be 642 million people living with the disease by 2040 and half as many again who will be not diagnosed. This means that pre-DM screening is a critical issue. Insulin resistance (IR) has emerged as a major pathophysiological factor in the development and progression of DM since it is evident in susceptible individuals at the early stages of DM, and particularly type 2 DM (T2DM). Therefore, assessment of IR *via* the homeostasis model assessment of IR (HOMA-IR) is a key index for the primary prevention of DM and is thus found in guidelines for screening of high-risk groups. However, the cut-off values of HOMA-IR differ for different races, ages, genders, diseases, complications, *etc*. due to the complexity of IR. This hampers the determination of specific cut-off values of HOMA-IR in different places and in different situations. China has not published an official index to gauge IR for primary prevention of T2DM in the diabetic and non-diabetic population except for children and adolescents ages 6-12 years. Hence, this article summarizes developments in research on IR, HOMA-IR, and pre-DM screening in order to provide a reference for optimal cut-off values of HOMA-IR for the diagnosis of DM in the Chinese population.**

Keywords: Insulin resistance; homeostasis model assessment of insulin resistance (HOMA-IR); diabetes mellitus type 2

1. Introduction

Diabetes mellitus (DM) has become prevalent with changes in lifestyle, threatening to reduce life expectancy for humans around the globe (*1,2*). Globally, there were a total of 382 million patients with DM in 2013 (*3*). The projections for the future constitute a dramatic call to countries and their governments. The International Diabetes Federation (IDF) has estimated that there will be 642 million people living with the disease by 2040 and half as many again who will be living with undiagnosed DM, unknowingly at risk from its disabling, life-threatening complications (*4*). This means that pre-DM screening is a critical issue.

In addition, DM appears to be increasing rapidly in China. The overall prevalence of DM was estimated to be 11.6% in the Chinese adult population in 2010, which is considerably higher than its prevalence of less than 1% in 1980 (*5*). Recent studies in China have found that there were 92.4 million persons with DM and 148.2 million persons with pre-DM in 2013, and 60% of patients were not diagnosed (*6*).

Type 2 DM (T2DM) is a complex, polygenetic hereditary disease associated with both heritable and environmental factors. Insulin resistance (IR) is a major pathophysiological factor in the development and progression of DM, and IR is also evident in a variety

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of metabolic diseases, such as obesity, hypertension, and dyslipidemia (*7-10*). Epidemiological studies have shown that about 25% of the population has IR and that the prevalence of IR is more than 80% in patients with T2DM (*11-13*). Guidelines for primary prevention of T2DM should identify categories of increased risk for DM (pre-DM), but many do not include the cut-off values of IR (*14,15*).

IR is now used as a screening index for the primary prevention of DM. This article examines developments in research on IR. Determining optimal HOMA-IR cut-off values would facilitate the diagnosis of DM in the Chinese population.

2. IR and DM

2.1. *Definition*

The concept of IR was proposed as early as 1936 and is generally defined as reduced biological action of insulin, such as inhibition of hepatic glucose production and insulin-mediated glucose disposal (*16,17)*.

IR increases the incidence of metabolic syndrome (MS), which has emerged as a major pathophysiological factor in the development and progression of many common non-communicable diseases, including T2DM, polycystic ovary disease, dyslipidemia, hypertension, cardiovascular disease and obesity (*18-20*).

2.2. *Inducement of IR*

2.2.1. *Diet*

IR commonly coexists with obesity, which may because dietary fat has long been implicated as a driver of IR. Recent research has suggested that the intake of simple sugars, and particularly fructose, is also a factor that contributes to IR (*21*). Another possible explanation is that both IR and obesity often have the same cause, systematic overeating. Systematic overeating has the potential to lead to IR and obesity due to repeated administration of excess levels of glucose, which stimulate insulin secretion; excess levels of fructose, which raise triglyceride levels in the bloodstream; and fats, which may be readily absorbed by adipose cells and up as fatty tissue in a hypercaloric diet.

2.2.2. *DM*

Recent research and experimentation have uncovered a non-obesity related connection between IR and T2DM (*22*). Increased insulin sensitivity or remission of T2DM has long been noted in patients who have undergone some form of bariatric surgery (*23*). Increased insulin sensitivity or remission of T2DM has also been noted in diabetic or insulin-resistant non-obese rats that have had their duodenum surgically removed (*24*).

2.2.3. *Hepatitis C virus (HCV)*

HCV also makes people three to four times more likely to develop IR and T2DM. In addition, people infected with the HCV who develop DM probably have susceptible insulin-producing cells and probably would have developed DM anyway, but much later in life. The extra IR caused by HCV apparently brings on DM at age 35 or 40, instead of 65 or 70 (*25*).

2.2.4. *Sedentary lifestyle*

A sedentary lifestyle increases the likelihood of developing IR (*26*). For each 500 kcal/week increment in energy expenditure as a result of physical activity, the lifetime risk of T2DM decreases by 6% (*27*). According to one study, vigorous exercise at least once a week reduced the risk of T2DM in women by 33% (*28*).

2.3. *Pathogenesis of DM*

Reaven proposed a model for DM caused by IR whereby IR manifests in susceptible individuals in the early stages of DM, and particularly in T2DM. Resistance to insulin-stimulated glucose uptake is evident in most patients with impaired glucose tolerance (IGT) or non-insulin-dependent DM (NIDDM) and in 0-25% of non-obese individuals with normal oral glucose tolerance (*29,30*).

The pathogenesis of DM is as follows: *i*) When food containing carbohydrates is consumed, the digestive system breaks carbohydrates down into sugar that then enters the blood. As blood sugar levels rise, the hormone insulin is secreted by the islets of Langerhans in the pancreas to prompt cells to absorb sugar for energy or storage; *ii*) Adverse environmental factors or disease can cause cells to fail to respond to the normal actions of insulin, resulting in IR; *iii*) Once IR develops and the body produces insulin, the body's cells fail to respond to insulin and are unable to use it effectively (IGT); *iv*) When the condition develops further, apoptosis of islet cells occurs and glucose metabolism is disrupted, leading to clinical DM (*31*).

3. Calculation of IR and its use in the primary prevention of T2DM

3.1. *Calculation of IR*

The Homeostasis Model Assessment of IR (HOMA-IR) has proved to be a robust tool for the assessment of IR and is the index of IR that is most widely used in large population studies (*32-34*). The HOMA of β-cell function and IR was first described in 1985 (*35,36*). HOMA-IR and HOMA-%β are determined using the following simplified equations:

HOMA-IR = (FPI × FPG) / 22.5;

HOMA-%β = (20 × FPI) / (FPG - 3.5)

Table 1. Main cut-off values of HOMA-IR in recent literature (sample size ≥ 1000)

Location and time	Sample size	Population characteristics	Threshold value	Criteria	References
Sweden, 2000	*n* = 4,816	Healthy population	2.0	75th percentile	(*43*)
France, 2002	*n* = 1,153	Age: 35 - 64; Healthy population	3.8	75th percentile	(*44*)
Caucasus, 2006	*n* = 1,156	Rural population; non-diabetic	2.29	75th percentile	(*45*)
Brazil, 2006	*n* = 1,317	Age: 40 ± 12 years; BMI: 34 ± 10 kg/m^2	2.77	90th percentile	(*46*)
U.S., 2008	*n* = 2,804	Age ≥ 20; normal BMI and fasting glucose	2.73	66th percentile	(*47*)
Iran, 2010	*n* = 3,071	Adult individuals; ages: 25-64 years	3.875	ROC curve	(*48*)
Iran, 2011	*n* = 1,036	Women of reproductive age	2.63	95th percentile	(*49*)
Japan, 2012	*n* = 6,868	Non-diabetic subjects	1.7	ROC	(*50*)
China, 2013	*n* = 3,203	Ages: 6-18 years (children and adolescents)	3.0	95th percentile	(*51*)
Portugal, 2014	*n* = 1,784	Non-diabetic individuals in a Cardiology ward; BMI < 25 Kg/m^2; FPG < 100 mg/dL	2.33	90th percentile	(*52*)

Here, FPI is the fasting plasma insulin concentration (mU/L) and FPG is fasting plasma glucose (mmol/L) (*37*).

3.2. *Use of HOMA-IR in the primary prevention of T2DM*

Primary prevention of T2DM means preventing T2DM from developing or identifying high-risk groups and taking steps to mitigate T2DM. Generally, categories of increased risk for DM (pre-DM) in guidelines on DM are: *i*) FPG of 100 mg/dL (5.6 mmol/L) to 125 mg/dL (6.9 mmol/L) (IFG); or *ii*) 2-h plasma glucose in the 75-g oral glucose tolerance test (OGTT) of 140 mg/dL (7.8 mmol/L) to 199 mg/dL (11.0 mmol/L) (IGT); or *iii*) an A1C of 5.7-6.4% (*38*).

Testing of asymptomatic people to detect T2DM and assess the future risk of DM should be considered for adults of any age who are overweight or obese (BMI ≥ 25 kg/m^2) and who have one or more additional risk factors for DM according to the following indexes: *i*) physical inactivity *ii*) a first-degree relative with DM; *iii*) high-risk race/ethnicity; *iv*) women who delivered a baby weighing 9 lb or who were diagnosed with gestational DM; *v*) hypertension; *vi*) an HDL cholesterol level of 35 mg/dL (0.90 mmol/L) and/or a triglyceride level of 250 mg/dL (2.82 mmol/L); *vii*) women with polycystic ovary syndrome (PCOS); *viii*) A1C ≥ 5.7%, impaired glucose tolerance (IGT), or impaired fasting glucose (IFG) on previous testing; *ix*) other clinical conditions associated with IR (*e.g.*, severe obesity); *x*) a history of cardiovascular disease (CVD). Testing of asymptomatic people who lack these risk factors should begin at age 45.

Although the major role of IR is cited in point *ix*) above, guidelines for diagnosis of DM have not defined the cut-off values of IR for high-risk groups (*39*).

3.3. *Principles for determination of HOMA-IR cutoff values*

The use of predetermined HOMA-IR cut-off values to identify individuals with IR leads to certain issues. The determination of HOMA-IR cut-off values affects the identification of IR and healthcare management for individuals of different genders, ages, or races and individuals with different diseases and complications (*40,41*).

Although IR is usually defined as a value greater than the 75th percentile value for non-diabetic subjects according to the World Health Organization (WHO) (*42*), the cut-off values reported in the literature vary widely (Table 1) (*43-52*).

4. Prospects for the future

As this review has elaborated, IR develops in susceptible individuals in the early stages of DM, and particularly T2DM. IR can be measured using HOMA-IR. At the present time, however, the glucose clamp technique is used to quantify beta-cell sensitivity to glucose and insulin (*53*). The glucose clamp technique offers a highly reproducible method of assessing sensitivity to glucose and tissue sensitivity to insulin, but it is complex and difficult to use. Thus, HOMA-IR tends to be a more convenient and efficient way to measure IR even though it is calculated solely from the FPI and PFG.

Using HOMA-IR to diagnose DM is an unscientific approach because DM can be caused by IR as well as excess insulin. That said, IR is clearly associated with

a pre-diabetic state. The cut-off values of HOMA-IR differ for different races, ages, genders, diseases, complications, *etc*. (*54*). An individual with a high HOMA-IR should nevertheless seek medical advice, exercise, and change his or her lifestyle, regardless of whether or not the individual has a metabolic disease. The cut-off values of HOMA-IR need to be examined in non-diabetic subjects in order to devise a standard for the primary prevention of DM.

At the present time, China has not published an official index to gauge IR for primary prevention of T2DM in the diabetic and non-diabetic population except for children and adolescents ages 6-12 years. The current review should provide a reference for the control of T2DM (*55*).

The current study has several limitations. First and foremost, a specific HOMA-IR cut-off value has not been calculated based on gender, age, race, *etc*., and only reference values are indicated. Second, variations in cut-off values of HOMA-IR in different countries have not been analyzed.

5. Conclusion

In conclusion, this article has defined DM, it has explained how DM can be induced, and it has described the role of IR in the pathogenesis of DM. This article has also summarized developments in research on IR and it has emphasized the significance of primary prevention of T2DM. Different HOMA-IR values for different races, ages, genders, diseases, complications, *etc*. are described for use in primary prevention of DM. This article should provide a reference for optimal cut-off values of HOMA-IR for the diagnosis of DM in the Chinese population.

References

1. Zhao E, Zhang Y, Zeng X, Liu B. Association between maternal diabetes mellitus and the risk of congenital malformations: A meta-analysis of cohort studies. Drug Discov Ther. 2015; 9:274-281.
2. Lau CH, Muniandy S. Novel adiponectin-resistin (AR) and insulin resistance (IRAR) indexes are useful integrated diagnostic biomarkers for insulin resistance, type 2 diabetes and metabolic syndrome: A case control study. Cardiovasc Diabetol. 2011; 10:8.
3. Shi Y, Hu FB. The global implications of diabetes and cancer. Lancet. 2014; 383:1947-1948.
4. World Guide to BRIDGES 2015. IDF.
5. Xu Y, Wang L, He J, *et al*. Prevalence and control of diabetes in Chinese adults. JAMA. 2013; 310:948-959.
6. Yang W, Lu J, Weng J, *et al*. Prevalence of diabetes among men and women in China. N Engl J Med. 2010; 362:1090-1101.
7. Polyzos SA, Kountouras J, Zavos C, Deretzi G. The association between Helicobacter pylori infection and insulin resistance: A systematic review. Helicobacter. 2011; 16:79-88.
8. Asdie RH, Sa'adah, Jazakillah S, Sinorita H. Does insulin resistance correlate with routine blood examination? A review on erythrocytes of obese patients. Acta Med Indones. 2009; 41:66-69.
9. Roberts CK, Hevener AL, Barnard RJ. Metabolic syndrome and insulin resistance: Underlying causes and modification by exercise training. Compr Physiol. 2013; 3:1-58.
10. Capasso I, Esposito E, Pentimalli F, *et al*. Homeostasis model assessment to detect insulin resistance and identify patients at high risk of breast cancer development: National Cancer Institute of Naples experience. J Exp Clin Cancer Res. 2013; 32:14.
11. Zhang M, Hu T, Zhang S, Zhou L. Associations of different adipose tissue depots with insulin resistance: A systematic review and meta-analysis of observational studies. Sci Rep. 2015; 5:18495.
12. Ghasemi A, Tohidi M, Derakhshan A, Hasheminia M, Azizi F, Hadaegh F. Cut-off points of homeostasis model assessment of insulin resistance, beta-cell function, and fasting serum insulin to identify future type 2 diabetes: Tehran Lipid and Glucose Study. Acta Diabetol. 2015; 52:905-915.
13. Yi KH, Hwang JS, Kim EY, Lee SH, Kim DH, Lim JS. Prevalence of insulin resistance and cardiometabolic risk in Korean children and adolescents: A population-based study. Diabetes Res Clin Pract. 2014; 103:106-113.
14. Kim HJ, Choi EY, Park EW, Cheong YS, Lee HY, Kim JH. The utility of HbA1c as a diagnostic criterion of diabetes. Korean J Fam Med. 2011; 32:383-389.
15. Li SY, Wang SY, Li J, Sun K, Zhang Z, Cao GL. The value of HbA1c for diagnosing type 2 diabetes mellitus between Chinese Uyghurs and Hans in Xinjiang. Int J Clin Exp Med. 2015; 8:11352-11355.
16. Himsworth HP. Diabetes mellitus: Its differentiation into insulin-sensitive and insulin-insensitive types. Lancet. 1936; 227:127-130.
17. Alebić MŠ, Bulum T, Stojanović N, Duvnjak L. Definition of insulin resistance using the homeostasis model assessment (HOMA-IR) in IVF patients diagnosed with polycystic ovary syndrome (PCOS) according to the Rotterdam criteria. Endocrine. 2014; 47:625-630.
18. Singh Y, Garg MK, Tandon N, Marwaha RK. A study of insulin resistance by HOMA-IR and its cut-off value to identify metabolic syndrome in urban Indian adolescents. J Clin Res Pediatr Endocrinol. 2013; 5:245-251.
19. Hernandez AV, Guarnizo M, Miranda Y, Pasupuleti V, Deshpande A, Paico S, Lenti H, Ganoza S, Montalvo L, Thota P, Lazaro H. Association between insulin resistance and breast carcinoma: A systematic review and meta-analysis. PLoS One. 2014; 9:e99317.
20. Yang Y, Wei RB, Xing Y, Tang L, Zheng XY, Wang ZC, Gao YW, Li MX, Chen XM. A meta-analysis of the effect of angiotensin receptor blockers and calcium channel blockers on blood pressure, glycemia and the HOMA-IR index in non-diabetic patients. Metabolism. 2013; 62:1858-1866.
21. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature. 2006; 444:840-846.
22. Kozawa J, Kitamura T, Nishizawa H, Yasuda T, Maeda N, Otsuki M, Okita K, Iwahashi H, Kaneto H, Funahashi T, Imagawa A, Shimomura I. Dipeptidyl peptidase-4 inhibitors are effective in Japanese type 2 diabetic patients with sustained endogenous insulin-

secreting capacity, a higher body mass index and insulin resistance. J Diabetes Investig. 2013; 4:190-194.
23. Rao RS, Yanagisawa R, Kini S. Insulin resistance and bariatric surgery. Obes Rev. 2012; 13:316-228.
24. Wongwananuruk T, Rattanachaiyanont M, Leerasiri P, Indhavivadhana S, Techatraisak K, Angsuwathana S, Tanmahasamut P, Dangrat C. The usefulness of Homeostatic Measurement Assessment-Insulin Resistance (HOMA-IR) for detection of glucose intolerance in Thai women of reproductive age with polycystic ovary syndrome. Int J Endocrinol. 2012; 571035.
25. Wongwananuruk T, Rattanachaiyanont M, Leerasiri P, Indhavivadhana S, Techatraisak K, Angsuwathana S, Tanmahasamut P, Dangrat C. Chronic hepatitis C is associated with peripheral rather than hepatic insulin resistance. Gastroenterology. 2010; 138:932-941.
26. Mayer-Davis EJ, D'Agostino R Jr, Karter AJ, Haffner SM, Rewers MJ, Saad M, Bergman RN. Intensity and amount of physical activity in relation to insulin sensitivity: The Insulin Resistance Atherosclerosis Study. JAMA. 1998; 279:669-674.
27. Helmrich SP, Ragland DR, Leung RW, Paffenbarger RS Jr. Physical activity and reduced occurrence of non-insulin-dependent diabetes mellitus. N Engl J Med. 1991; 325:147-152.
28. Manson JE, Rimm EB, Stampfer MJ, Colditz GA, Willett WC, Krolewski AS, Rosner B, Hennekens CH, Speizer FE. Physical activity and incidence of non-insulin-dependent diabetes mellitus in women. Lancet. 1991; 338:774-778.
29. Reaven GM. Banting Lecture 1988. Role of insulin resistance in human disease. 1988. Nutrition. 1997; 13:65-66.
30. Ismail NA, Kasim MM, Noor Aizuddin A, Umar NA. Homeostatic indices of insulin resistance among gestational diabetics in anticipating pregnancy complications. Gynecol Endocrinol. 2013; 29:691-694.
31. Olefsky JM, Kolterman OG. Mechanisms of insulin resistance in obesity and noninsulin-dependent (type II) diabetes. Am J Med. 1981; 70:151-168.
32. Lann D, LeRoith D. Insulin resistance as the underlying cause for the metabolic syndrome. Med Clin North Am. 2007; 91:1063-1077.
33. Antuna-Puente B, Disse E, Rabasa-Lhoret R, Laville M, Capeau J, Bastard JP: How can we measure insulin sensitivity/resistance? Diabetes Metab. 2011; 37:179-188.
34. Mojiminiyi OA, Abdella NA. Effect of homeostasis model assessment computational method on the definition and associations of insulin resistance. Clin Chem Lab Med. 2010; 48:1629-1634.
35. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: Insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985; 28:412- 419.
36. Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. Diabetes Care. 2004; 27:1487-1495.
37. Haffner SM, Kennedy E, Gonzalez C, Stern MP, Miettinen H. A prospective analysis of the Homa model. The Mexico City diabetes study. Diabetes Care. 1996; 19:1138-1141.
38. American Diabetes Association. Standards of Medical Care in Diabetes – 2015. Diabetes Care. 2015; 38:S1.
39. Ozcimen EE, Uckuyu A, Ciftci FC, Yanik FF, Bakar C. Diagnosis of gestational diabetes mellitus by use of the homeostasis model assessment-insulin resistance index in the first trimester. Gynecol Endocrinol. 2008; 24:224-229.
40. Gayoso-Diz P, Otero-González A, Rodriguez-Alvarez MX, Gude F, García F, De Francisco A, Quintela AG. Insulin resistance (HOMA-IR) cut-off values and the metabolic syndrome in a general adult population: Effect of gender and age: EPIRCE cross-sectional study. BMC Endocr Disord. 2013; 13:47.
41. Quinn SM, Baur LA, Garnett SP, Cowell CT. Treatment of clinical insulin resistance in children: A systematic review. Obes Rev. 2010; 11:722-730.
42. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med. 1998; 15:539-553.
43. Hedblad B, Nilsson P, Janzon L, Berglund G. Relation between insulin resistance and carotid intima-media thickness and stenosis in non-diabetic subjects. Results from a cross-sectional study in Malmo, Sweden. Diabet Med. 2000; 17:299-307.
44. Marques-Vidal P, Mazoyer E, Bongard V, Gourdy P, Ruidavets JB, Drouet L, Ferrières J. Prevalence of insulin resistance syndrome in Southwestern France and its relationship with inflammatory and haemostatic markers. Diabetes Care. 2002; 25:1371-1377.
45. Radikova Z, Koska J, Huckova M, Ksinantova L, Imrich R, Vigas M, Trnovec T, Langer P, Sebokova E, Klimes I. Insulin sensitivity indices: A proposal of cut-off points for simple identification of insulin-resistant subjects. Exp Clin Endocrinol Diabetes. 2006; 114:249-256.
46. Geloneze B, Repetto EM, Geloneze SR, Tambascia MA, Ermetice MN. The threshold value for insulin resistance (HOMA-IR) in an admixture population IR in the Brazilian metabolic syndrome study. Diabetes Res Clin Pract. 2006; 72:219-220.
47. Summer AE, Cowie CC. Ethnic differences in the ability of triglyceride levels to identify insulin resistance. Atherosclerosis. 2008; 196:696-703.
48. Esteghamati A, Ashraf H, Khalilzadeh O, Zandieh A, Nakhjavani M, Rashidi A, Haghazali M, Asgari F. Optimal cut-off of homeostasis model assessment of insulin resistance (HOMA-IR) for the diagnosis of metabolic syndrome: Third national surveillance of risk factors of non-communicable diseases in Iran (SuRFNCD-2007). Nutr Metab (Lond). 2010; 7:7-26.
49. Zadeh-Vakili A, Tehrani FR, Hosseinpanah F. Waist circumference and insulin resistance: A community based cross sectional study on reproductive aged Iranian women. Diabetol Metab Syndr. 2011; 3:18.
50. Yamada C, Moriyama K, Takahashi E. Optimal cut-off point for homeostasis model assessment of insulin resistance to discriminate metabolic syndrome in non-diabetic Japanese subjects. J Diabetes Investig. 2012; 3:384-387.
51. Yin J, Li M, Xu L, Wang Y, Cheng H, Zhao X, Mi J. Insulin resistance determined by Homeostasis Model Assessment (HOMA) and associations with metabolic syndrome among Chinese children and teenagers. Diabetol Metab Syndr. 2013; 5:71.
52. Timóteo AT, Miranda F, Carmo MM, Ferreira RC. Optimal cut-off value for homeostasis model assessment (HOMA) index of insulin-resistance in a population of patients admitted electively in a Portuguese cardiology ward. Acta Med Port. 2014; 27:473-479.

53. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: A method for quantifying insulin secretion and resistance. Am J Physiol. 1979; 237:214-223.
54. Nadeem A, Naveed AK, Hussain MM, Raza SI. Cut-off values of anthropometric indices to determine insulin resistance in Pakistani adults. J Pak Med Assoc. 2013; 63:1220-1225.
55. Stabe C, Vasques AC, Lima MM, Tambascia MA, Pareja JC, Yamanaka A, Geloneze B. Neck circumference as a simple tool for identifying the metabolic syndrome and insulin resistance: Results from the Brazilian Metabolic Syndrome Study. Clin Endocrinol (Oxf). 2013; 78:874-881.

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Original Article

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Inhibitory effects of several saturated fatty acids and their related fatty alcohols on the growth of *Candida albicans*

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Summary **We examined the effect of 5 saturated fatty acids and their related alcohols on the growth of *Candida albicans*. The inhibitory effects of these compounds against the yeast and hyphal growth forms of *C. albicans* were examined using the modified NCCLS method and crystal violet staining, respectively. Among these compounds, capric acid inhibited both types of growth at the lowest concentration. The IC_{80}, *i.e.*, the concentration at which the compounds reduced the growth of *C. albicans* by 80% in comparison with the growth of control cells, of capric acid for the hyphal growth of this fungus, which is indispensable for its mucosal invasion, was 16.7 μM. These fatty acids, including capric acid, have an unpleasant smell, which may limit their therapeutic use. To test them at reduced concentrations, the combined effect of these fatty acids and oligonol, a depolymerized polyphenol, was evaluated *in vitro*. These combinations showed potent synergistic inhibition of hyphal growth [fractional inhibitory concentration (FIC) index = 0.319]. Our results demonstrated that capric acid combined with oligonol could be used as an effective anti-*Candida* compound. It may be a candidate prophylactic or therapeutic tool against mucosal *Candida* infection.**

Keywords: Medium-chain fatty acid, capric acid, oligonol, *Candida albicans*

1. Introduction

Candida albicans, a dimorphic fungus, is a member of the oral and intestinal microbial flora in healthy human individuals. Its excessive growth can cause pathological symptoms such as oral, esophageal, vaginal, or systemic candidiasis (*1,2*). Recently, it was suggested that heavy colonization by *C. albicans* predisposes to various types of inflammatory diseases (*3*). There are several types of foods that can control *Candida* growth *in vitro* and *in vivo*, for example, lemongrass, green tea, and cassia (*4*). Consuming foods with anti-*Candida* activity may prevent the excessive growth of *C. albicans*. It has been reported that medium-chain fatty acids have anti-*Candida* activity (*5*). These fatty acids might be the functional food components for the improvement of symptoms related to *Candida* overgrowth. We have previously demonstrated that capric acid is an active component responsible for the anti-*Candida* activity of *Houttuynia cordata* (*6*).

In the present study, we systematically examined the effects of several saturated fatty acids and their related fatty alcohols on the growth of *C. albicans*. We demonstrated that capric acid could be used in anti-*Candida* treatment and might be a candidate prophylactic or therapeutic tool against mucosal *Candida* infection.

2. Materials and Methods

2.1. *C. albicans strain*

We used *C. albicans* strain TIMM1768, a clinically isolated serotype A strain (Teikyo University Institute of Medical Mycology, Tokyo, Japan).

2.2. *Medium-chain fatty acids, their related fatty alcohols, and oligonol*

Medium-chain fatty acids and related fatty alcohols were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). They were dissolved in dimethyl sulfoxide (DMSO) at 10% w/w before dilution with

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RPMI-1640 medium (RP medium). Oligonol, a low-molecular-weight polyphenol formulation derived from lychee fruit (Amino Up Chemical Co., Ltd.), was diluted with RP medium for *in vitro* experiments.

2.3. *Inhibitory effects of the compounds against C. albicans yeast growth*

The inhibitory effects of several saturated fatty acids and their related fatty alcohols against *C. albicans* yeast growth were determined using the microbroth dilution assay recommended by NCCLS M-27-A (7). *C. albicans* cells were cultured in YPG medium (1% Bacto-peptone, 0.5% yeast extract, 2% glucose, pH 6.5) for 16 h at 37°C with shaking at 38 rpm. The cells were collected and washed twice with RP medium, and the cell suspension was prepared in the same medium at 1×10^4 cells/mL. Medium-chain fatty acids and their related fatty alcohols in DMSO and DMSO control samples were diluted with RP medium. Mixtures of 100 µL of *Candida* cell suspension and 100 µL of various compound dilutions in DMSO (or control) were placed in a 96-well microplate. The microplate was incubated for 24 h at 30°C. Then, the minimum inhibitory concentration (MIC) values were determined.

2.4. *Inhibitory effects of the compounds against C. albicans hyphal growth*

RP medium supplemented with 2.5% heat-inactivated fetal calf serum, 20 mM HEPES, 2 mM L-glutamine, and 16 mM sodium hydrogen carbonate (pH 7.0) was used as the hyphal growth-promoting medium for *C. albicans*. *C. albicans* suspension was prepared at 5×10^3 cells/mL. Each well of a 96-well flat-bottom microplate received a mixture of 100 µL of *Candida* suspension, 100 µL of fatty acid or fatty alcohol preparations, or 50 µL of fatty acid or fatty alcohol preparation or oligonol preparations. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 15 h. To determine the extent of *C. albicans* hyphal growth, the crystal violet (CV) staining assay was performed as described previously (*8*). In brief, the medium from the wells was discarded and the adhering *Candida* mycelia were sterilized with 70% ethanol. The mycelia were stained with 0.01% CV and washed with water. The microplates were dried and 150 µL of isopropanol containing 0.04 N HCl and 50 µL of 0.25% sodium dodecyl sulfate were added to the wells and mixed. The absorbance at 620 nm (triplicate samples) was measured spectrophotometrically. MIC was defined as the lowest compound concentration that reduced growth by 80% or 85% in comparison with the growth in the drug-free well.

To analyze the combined anti-*Candida* activities, the fractional inhibitory concentration (FIC) index was calculated as follows: FIC = [(A)/MICA] + [(B)/MICB], where MICA and MICB are the MICs of samples A and B, respectively, determined separately. (A) and (B) are the concentrations of the samples in combination, respectively, in all of the wells corresponding to an MIC (isoeffective combinations) (*9*). FIC indices were used to characterize antibiotic interactions as follows: synergy, FIC index ≤ 0.5; additivity, 0.5 < FIC index < 1; indifference, 1 < FIC index ≤ 4; and antagonism, FIC index > 4.

3. Results

3.1. *Inhibitory effects of saturated fatty acids and their related fatty alcohols on C. albicans yeast growth*

The inhibitory effects of 5 saturated fatty acids and 4 fatty alcohols against *C. albicans* yeast growth were examined using the modified NCCLS method (Table 1). The MICs of octanoic acid, capric acid, and lauric acid against yeast growth were 34.7 mM, 29.0 mM, and 49.9 mM, respectively. However, $C_{8\text{-}12}$ alcohols 1-octanol, decanol, and dodecanol did not affect the growth of *Candida* cells at concentrations below 200 mM. C_4 acids and C_{14} acid and alcohol (sodium butyrate, myristic acid, and 1-tetradecanol) did not significantly affect yeast growth at concentrations below 100 mM. Thus, medium-chain fatty acids showed a stronger inhibitory effect than short- and long-chain fatty acids. The inhibitory properties of related alcohols were weak.

3.2. *Inhibitory effects of saturated fatty acids and fatty alcohols on C. albicans hyphal growth*

The inhibitory effects of various saturated fatty acids and fatty alcohols against the growing hyphae of *C. albicans* were examined using the CV staining method (Table 2). Most of the tested compounds significantly inhibited the hyphal growth of the fungus at very low concentrations.

The inhibitory effects of 5 saturated fatty acids and 4 fatty alcohols were compared in terms of their IC_{80} values, *i.e.*, the concentration at which the compounds reduced the growth of *C. albicans* by 80% in comparison with the growth of control cells. The IC_{80} of capric acid and lauric acid was 16.7 µM and 61.0 µM, respectively. These inhibitory concentrations were approximately 1/1,000 of the MIC for yeast growth (Tables 1 and 2). However, 205 µM octanoic acid was needed for 80% inhibition of *C. albicans* hyphal growth. The IC_{80} of 1-octanol was almost the same as that of octanoic acid (175 µM). The IC_{80} of decanol (204 µM) and dodecanol (401 µM) was approximately 10 times higher than that of capric and lauric acid. The IC_{80} of myristic acid was 833 µM. Sodium butyrate did not inhibit *Candida* hyphal growth at concentrations below 1.82×10^3 µM.

These results show that the inhibitory effects of

Table 1. Effect of saturated fatty acids and their related fatty alcohols on the total growth

C	Fatty acid	MIC (mM)	Fatty acid alcohol	MIC (mM)
4	Sodium n-Butyrate	363	–	–
8	n-Octanoic acid	34.7	1-Octanol	307
10	Capric acid	29.0	Decanol	> 253
12	Lauric acid	49.9	Dodecanol	> 215
14	Myristic acid	> 175	1-Tetradecanol	> 187

Activities were measured using the modified NCCLS method as described in the Materials and Methods section. The minimum inhibitory concentration (MIC) against *Candida* growth is shown.

Table 2. Effect of several saturated fatty acids and their related fatty alcohols on *C. albicans* hyphal growth

C	Fatty acid	IC_{80} (μM)	Fatty alcohol	IC_{80} (μM)
4	Sodium n-Butyrate	$> 1.82 \times 10^3$	–	–
8	n-Octanoic acid	205	1-Octanol	175
10	Capric acid	16.7	Decanol	204
12	Lauric acid	61.0	Dodecanol	401
14	Myristic acid	833	1-Tetradecanol	> 930

Activities were measured using the CV staining method as described in the Materials and Methods section. The concentration causing 80% inhibition (IC_{80}) of *Candida* hyphal growth is indicated.

Table 3. IC_{85} and FIC index for medium-chain fatty acids or their related alcohols in combination with oligonol against *Candida* hyphal growth

Items	IC_{85} (μM)	IC_{85} with Oligonol 62.5 μg/mL	FIC index
Capric acid	14.5	2.90	0.319
Lauric acid	32.0	13.5	0.541
Decanol	106	68.8	0.800
Dodecanol	440	123	0.432

Activities were measured using the CV staining method. The concentration causing 85% inhibition (IC_{85}) of *Candida* hyphal growth using a combination of medium-chain fatty acids or alcohols and/or oligonol (62.5 μg/ml) is indicated. The FIC index was calculated as described in the Materials and Methods section.

C_{10} and C_{12} acids against *Candida* hyphal growth were exceptionally strong compared with the inhibitory effects against *Candida* yeast growth. This inhibition was approximately 10 times stronger than the effect of C_{10} and C_{12} alcohols.

3.3. *Inhibition of C. albicans hyphal growth by saturated fatty acids or their related fatty alcohols in combination with a low-molecular-weight polyphenol*

It has been reported that a combination of capric acid and terpinen-4-ol, a major component of tea tree oil, inhibits *Candida* hyphal growth synergistically (*10*). We have also reported that oligonol, a low-molecular-weight polyphenol formulation derived from lychee fruit, inhibits *Candida* hyphal growth (*11*). The preparation has attained a self-affirmed Generally Recognized as Safe (GRAS) status in the USA, which supports its safety as a food product. Here we examined the inhibitory effect of a combination of C_8-C_{12} acids or alcohols and oligonol against *C. albicans* hyphal growth. Their combined effect was evaluated in terms of reduction of the IC_{85} value and the FIC index (Table 3).

In the case of capric acid alone, 14.5 μM concentration was needed for 85% inhibition (IC_{85}) of *Candida* hyphal growth (Figure 1A). However, the IC_{85} of capric acid administered in combination with oligonol (62.5 μg/mL) decreased to approximately 1/5 of this value (2.90 μM) (Figure 1A). The IC_{85} of lauric acid when combined with oligonol was reduced to approximately half of the value obtained when it was used alone (13.5 μM). Figure 1B shows the concentrations of capric acid and oligonol in combination showing 85% inhibition of *Candida* growth. The curve, located under the dotted line, indicates that the combined effect was synergistic. The data in Table 3 shows that the combination of capric acid (3.50 μM) and oligonol (31.3 μg/mL) displayed synergistic activity (FIC index = 0.319). The FIC index of lauric acid with oligonol slightly exceeded 0.500. Using dodecanol alone, a concentration of 440 μM was needed for 85% inhibition of *Candida* hyphal growth (30 times higher than the IC_{85} of capric acid). The IC_{85} of dodecanol decreased to 1/4 on combination with oligonol; a synergistic effect was observed (FIC index = 0.432).

These results indicated that capric acid and dodecanol with oligonol effectively repressed *Candida* hyphal growth.

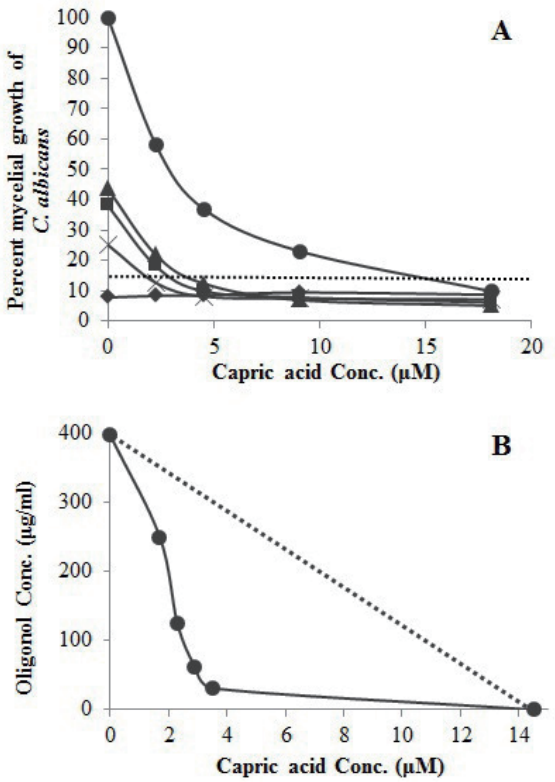

Figure 1. *Candida* growth in a medium containing various concentrations of capric acid and/or oligonol. (A) *C. albicans* cells (TIMM1768) were cultured in a medium containing a combination of the indicated concentrations of capric acid and 0 (●), 31.3 (▲), 62.5 (■), 250 (×), or 500 (♦) μg/mL of oligonol for 15 h (dotted line = IC_{85}). (B) Analysis of the combined effect. Each point represents the concentration of the combination of capric acid and oligonol causing 85% inhibition of *C. albicans* hyphal growth. If the combined effect was additive, the point for the combination would lie on the dotted line.

4. Discussion

It has been reported that a low concentration of capric acid inhibits *C. albicans* hyphal growth *in vitro* and that oral administration of approximately 10 mg/mL (50 μL) of capric acid protects mice from oral candidiasis (*12*). These data suggest that capric acid may be used as a functional food with anti-*Candida* activity. However, capric acid has a characteristic unpleasant smell; therefore, it might not be suitable for oral administration. To find a better candidate for oral use, we examined the anti-*Candida* activity of other fatty acids and their related alcohols. The results clearly showed that among the tested compounds, capric acid inhibited *C. albicans* yeast and hyphal growth at the lowest concentration. This result demonstrates that capric acid is the most suitable candidate for protection against mucosal candidiasis. Davis *et al.* (*13*) have reported that dodecanol (C_{12} alcohol) effectively represses *Candida* hyphal growth. Here we also confirmed that straight-chain fatty alcohols inhibited hyphal growth but their effective concentrations (C_{10}, C_{12}) were much higher than the required concentrations of the related carbonic acids (Table 2). Therefore, we speculated that the effects of decanol and dodecanol could be mediated by their metabolic acids, capric and lauric acid, respectively.

To decrease the effective doses of capric acid for anti-*Candida* function, the inhibitory effect of the combination of capric acid and oligonol, a low-molecular-weight polyphenol formulation derived from lychee fruits, was tested. Polyphenols are likely to be some of the best compounds for such combinations; they have antimicrobial activity not only against *C. albicans* but also against *Helicobacter pylori* (*14*), *Staphylococcus aureus*, and *Escherichia coli* O157:H7 (*15*). By combining capric acid and lauric acid with oligonol, their IC_{85} values for inhibition of *C. albicans* hyphal growth were lowered to 3-14 μM. This result suggests that these fatty acids can function as effective anti-*Candida* compounds in the presence of polyphenols.

It would be useful to find out whether medium-chain fatty acids affect *C. albicans* growth in the human digestive tract. The concentration of medium-chain fatty acids in the gastrointestinal tract has not been examined thoroughly. However, it has been reported that approximately 50% of the total amount of medium-chain fatty acids infused into the duodenum gradually moves into the blood circulation within 3 h (*16*). This observation suggests that the medium-chain fatty acids in the gastrointestinal tract maintain their concentration at significant levels at least for a 3-h period. In Japan, the daily intake of medium-chain fatty acids is approximately 0.2 g. If a meal contains 0.02 g (1/10 of the daily intake) of medium-chain fatty acids and it arrives in the 100-cm^3 duodenum, the concentration in the duodenum will be approximately 1 mM. In this study, 1 mM medium-chain fatty acids could not inhibit *C. albicans* yeast growth *in vitro* but inhibited hyphal growth. We consider that medium-chain fatty acids, perhaps as metabolites of glycerides, have the potential to elicit their anti-*Candida* activity in the duodenum or small intestine, particularly in the presence of polyphenols.

The mechanism of inhibition of *Candida* hyphal growth by the combination of capric acid and oligonol is not clear. However, the inhibitory effect of dodecanol and catechin in the *Candida* hyphal growth pathway has been partially explained. Dodecanol exerts its effect through a mechanism involving enhanced expression of the *C. albicans* hyphal repressor Sfl1p (*17*). Catechin inhibits *C. albicans* dimorphism by suppressing Cek1 phosphorylation and cAMP synthesis (*18*). In our experiments, the combination of dodecanol and oligonol showed a synergistic inhibitory effect on *C. albicans* hyphal growth. We can speculate that the synergistic inhibitory effect of capric acid and oligonol might reflect complex interactions at different points in the pathway of hyphal growth.

In this study, a very low concentration of capric acid inhibited *C. albicans* hyphal growth. The intake of some neutral fats, such as coconut oil, composed of medium-chain fatty acids may inhibit the overgrowth of *C. albicans* in the gut. Medium-chain fatty acids are the products of fat degradation by lipase in the gut. We found that coconut oil (500 μg/mL) was degraded by lipase within 30 min, and its 10-fold diluted solution inhibited approximately 50% of *Candida* hyphal growth (data not shown). Future studies should examine the role of foods containing medium-chain fatty acids in the dynamic regulation of the ecology of *C. albicans* in our intestinal ducts, particularly in combination with other vegetable foods containing polyphenols.

References

1. Odds FC. A Review and Bibliography. *Candida* and candidosis: 4-129, Bailliere Tindale, London, 1988.
2. Dongari-Bagtzoglou A, Dwivedi P, Ioannidou E, Shaqman M, Hull D, Burleson J. Oral *Candida* infection and colonization in solid organ transplant recipients. Oral Microbiol Immunol. 2009; 24:249-254.
3. Abe S, Takizawa T. Mucosal *Candida* infection and its pathological effects on various inflammatory diseases. Med Mycol Res. 2014; 5:11-18.
4. Taguchi Y. Oral health care by utilizing food function. Med Mycol J. 2014; 55:J143-149.
5. Bergsson G, Arnfinnsson J, Steingrímsson O, Thormar H. *In vitro* killing of *Candida albicans* by fatty acids and monoglycerides. Antimicrob Agents Chemother. 2001; 45:3209-3212.
6. Inouye S, Takahashi M, Abe S. Inhibitory activity of hydrosols prepared from 18 Japanese herbs of weak aromatic flavor against filamentous formation and growth of *Candida albicans*. Med Mycol J. 2012; 53:33-40.
7. National Committee for Clinical Laboratory Standards: Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved Standard. NCCLS Document M27-A, NCCLS 17(9), Vallanova, Pa, 1997.
8. Abe S, Satoh T, Tokuda Y, Tansho S, Yamaguchi H. A rapid colorimetric assay for determination of leukocyte-mediated inhibition of mycelial growth of *Candida albicans*. Microbiol Immnol. 1994; 38:385-388.
9. Eliopoulos GM, Moellering RC. Antimicrobial combinations in Antibiotics in laboratory medicine, ed Lorian V. (The Williams & Wilkins Co. Baltimore, Md), 3rd ed.: 432-492, 1991.
10. Ninomiya K, Hayama K, Ishijima S, Takahashi M, Kurihara J, Abe S. Effects of inhibitory activity on mycelial growth of *Candida albicans* and therapy for murine oral candidiasis by the combined use of terpinen-4-ol and a middle-chain fatty acid, capric acid. Yakugaku Zasshi. 2013; 133:133-140.
11. Hayama K, Ishibashi H, Kitadate K, Yamazaki M, Abe S. Therapeutic effect of oligonol, a low-molecular polyphenol formulation derived from lychee fruits on murine oral candidiasis. Nihon Ishinkin Gakkai Zasshi. 2010; 51:137-142.
12. Takahashi M, Inoue S, Hayama K, Ninomiya K, Abe S. Inhibition of *Candida* mycelia growth by a medium chain fatty acids, capric acid in vitro and its therapeutic efficacy in murine oral candidiasis. Med Mycol J. 2012; 53:255-261.
13. Davis-Hanna A, Piispanen AE, Stateva LI, Hogan DA. Farnesol and dodecanol effects on the *Candida albicans* Ras1-cAMP signalling pathway and the regulation of morphogenesis. Mol Microbiol. 2008; 67:47-62.
14. Ankolekar C, Johnson D, Pinto Mda S, Johnson K, Labbe R, Shetty K. Inhibitory potential of tea polyphenolics and influence of extraction time against *Helicobacter pylori* and lack of inhibition of beneficial lactic acid bacteria. J Med Food. 2011; 14:1321-1329.
15. Nakayama M, Shigemune N, Tsugukuni T, Jun H. Mechanism of the combined anti-bacterial effect of green tea extract and NaCl against *Staphylococcus aureus* and *Escherichia coli* O157:H7. Food Control. 2012; 25:225-232.
16. Guillot E, Vaugelade P, Lemarchal P, Rérat A. Intestinal absorption and liver uptake of medium-chain fatty acids in non-anaesthetized pigs. Br J Nutr. 1993; 69:431-442.
17. Hall RA, Turner KJ, Chaloupka J, Cottier F, De Sordi L, Sanglard D, Levin LR, Buck J, Mühlschlegel FA. The quorum-sensing molecules farnesol/homoserine lactone and dodecanol operate via distinct modes of action in *Candida albicans*. Eukaryot Cell. 2011; 10:1034-1042.
18. Saito H, Tamura M, Imai K, Ishigami T, Ochiai K. Catechin inhibits *Candida albicans* dimorphism by disrupting Cek1 phosphorylation and cAMP synthesis. Microb Pathog. 2013; 56:16-20.

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Original Article

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Comparison of compounds of three *Rubus* species and their antioxidant activity

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Summary ***Rubus amabilis*, *Rubus niveus Thunb.*, and *Rubus sachalinensis* are three *Rubus* species that are alternatively found in Manubzhithang, a Tibetan medicine, in different areas of China. The current study analyzed HPLC/UV chromatograms and it compared compounds of these three *Rubus* species in contrast to reference substances such as 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside, procyanidin B4, and isovitexin-7-*O*-glucoside. The three *Rubus* species produced similar peaks in chromatograms. The antioxidant activity of the three *Rubus* species was determined using an assay for DPPH free radical scavenging activity. Results indicated that the three *Rubus* species extracts had almost the same level of free radical scavenging activity. Thus, findings indicated the rationality of substituting these species for one another as an ingredient in Manubzhithang.**

Keywords: *Rubus amabilis*, *Rubus niveus Thunb.*, *Rubus sachalinensis*, antioxidant activity, Tibetan medicine

1. Introduction

Rubus species (*Rosaceae*) such as *Rubus amabilis*, *Rubus niveus Thunb.*, and *Rubus sachalinensis* are often used in herbal medicines in China. These medicines contain various chemical compounds including polyphenolics, flavanols, flavonoid glycosides, pregnane glycosides, lignin glycosides, triterpene glucosyl esters, alkanols, anthocyanins, lignans, and tannins (*1,2*). These medicines can be used in traditional medicine as a radical scavenging agent (*3-5*), an anti-inflammatory, an anticonvulsant, a muscle relaxant, an antimicrobial, an antiphlogistic, an analgesic, an antidotal, or an antitumor agent (*6-9*).

Different areas substitute *Rubus amabilis*, *Rubus niveus Thunb.*, and *Rubus sachalinensis* for one another as a key ingredient in the traditional Tibetan medicine Manubzhithang. Manubzhithang is a powder that is used to make a broth, and this medicine has been used in Tibetan medicine for 1,300 years (*10*). Manubzhithang has exceptional ability to prevent and treat inflammatory disease, "depleted heat", gastrointestinal ulcers, and vascular angina. In northwest China, Manubzhithang consists of *R. amabilis*, *Inula helenium*, *Tinospora cordifolia (Willd.) Miers*, and *Zingiber officinale Rosc.* In southwest China, Manubzhithang consists of *R. niveus Thunb.*, *I. helenium*, *T. cordifolia (Willd.) Miers*, and *Z. officinale Rosc*. In Inner Mongolia and Xinjiang, China, Manubzhithang consists of *R. sachalinensis*, *I. helenium*, *T. cordifolia (Willd.) Miers*, and *Zingiber officinale Rosc.* or *Kaempferia galanga* L.

The current study analyzed the HPLC/UV chromatograms produced by *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis*. Compounds of the three *Rubus* species were compared to reference substances such as 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside (Compound 1), procyanidin B_4 (Compound 2), and isovitexin-7-*O*-glucoside (Compound 3). The antioxidant activity of the three *Rubus* species was also determined using an assay for DPPH free radical scavenging activity. To the extent known, the current study is the first to examine the rationality of substituting the three *Rubus* species for

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one another, based on their antioxidant activity, as a main ingredient in the traditional Tibetan medicine *Manubzhithang*.

2. Materials and Methods

2.1. *Plant materials and extract preparation*

Fresh *R. amabilis* was collected from the Northern Mountains National Forest in Huzhu, Qinghai, *R. niveus* Thunb. was collected from Nyingchi, Tibet, and *R. sachalinensis* was collected from the Dongwuqibaogeda Mountains in Inner Mongolia. Specimens of the plants were authenticated by the College of Chinese Medicine, Department of Chinese Medicinal Chemistry, Beijing University of Chinese Medicine. 2-deoxy-D-ribose, 2,2-dipheny-l-picrylhydrazyl (DPPH), and vitamin C were purchased from Sigma-Aldrich Co. (Shanghai, China). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Ethanol, phosphoric acid, and methanol were purchased from Beijing Hongxing Chemicals Co. (Beijing, China). All other reagents were of analytical grade unless otherwise noted.

The collected plants were transported to the laboratory and grouped accordingly. Plants were ground and sieved through a sieve with 0.25 mm mesh. Two g of each sample of *R. amabilis* powder was placed in a 250 mL round-bottomed flask and 50 mL of methanol was added. The contents of the flask were sonicated for 40 min. Afterwards, the contents were filtered and injected into an HPLC column. An additional 2.0 g of sample powder was also placed in a 250 mL round-bottomed flask and 50 mL of methanol was added. The contents of the flask were refluxed twice for 2 h (1 h for each reflux). Afterwards, the contents were filtered using a 0.45 mm membrane filter prior to injection into an HPLC column.

Two g of each sample of *R. amabilis* powder was placed in a 250 mL round bottomed flask and 50 mL of methanol or 95% ethanol was added. The contents of the flask were sonicated for 40 min. Afterwards, the contents were filtered and injected into an HPLC column.

2.2. *HPLC conditions*

HPLC was performed with a Waters high-pressure liquid chromatographic system equipped with a 1525 high-pressure gradient pump, a Kromasil 100-5C_{18} column (250 × 4.6mm, 5 μm), a 2998 PDA array detector, a 2707 automatic sampling injector, and a 2414 constant temperature system. The column temperature was set at 25°C. The mobile phase consisted of acetonitrile (A) and phosphoric acid:water (40:60) (B). (10-25% A; 90-75% B) (Table 1). Separation was performed by gradient elution at a total flow rate of 1.0 mL/min, a binary solvent mixture was used, and run time was adjusted to 30 min. The injection volume was 10 μL and elutes were detected at 210 nm. The standard stock solutions of isovitexin-7-*O*-glucoside, procyanidin B_4, and 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside were separated from *R. amabilis* in the laboratory.

Table 1. Gradient elution of the mobile phase

Time (min)	Acetonitrile (%)	Phosphoric acid:Water (0.4%)
0	10	90
5	15	85
30	25	75

2.3. *Comparison of compounds*

Standard stock solutions of isovitexin-7-*O*-glucoside, procyanidin B_4, and 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside were prepared in methanol. Chromatograms of the standard solutions, the standard extracts of *R. amabilis* (1.0g), and the extracts of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* (1.0g each) were compared.

2.4. *Determination of antioxidant activity in vitro*

Each plant was individually washed with tap water and deionized water. Afterwards, the plants were dried at 55.8°C until a constant weight was obtained. All of the samples (5.0 g of each) were ground into powder by using a glass mortar and ground samples were stored at -4°C until use. The dried and powdered plant material of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* (2.0 mg each), pure compounds of the three species, and isovitexin-7-*O*-glucoside, 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside, and procyanidin B_4 (2.0 mg each) were separately placed in a crucible, 100 μL of DMSO was added, and the mixture was stirred until all of the ingredients dissolved. Each sample was eluted with 900 μL of ethanol (100%) to yield a solution of 1.0 mg/mL. Different concentrations of samples (1.0, 0.50, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.0078125 mg/mL) were prepared using the 1.0-mg/mL solution. Ascorbic acid (vitamin C) was similarly prepared to serve as the positive control. Ethanol solutions of the samples (100 μL of 1.0, 0.50, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.0078125 mg/mL) or the positive control were mixed with a 100-μL solution of DPPH in a 96-well plate. One hundred μL of ethanol and 100 μl of DPPH ethanol served as negative control solutions. The mixtures were incubated at 37°C for 30 min and their absorbance was measured at 517 nm (DNM-9602G Micro-plate Reader, Beijing Aipu). The percentage inhibition of DPPH was calculated using the following equation:

Inhibition (%) = $[1 – (A_i – A_j) /A_c] \times 100\%$.

Here, A_c is the absorbance of the negative control (Ethanol solution of DPPH without test sample) and A_i is the absorbance of a mixture of a sample and DPPH at 517 nm. A_j is absorbance of the sample without DPPH at 517 nm. Half-maximal inhibition concentrations (IC_{50} values) were calculated from linear regression plots, where the abscissa represented the concentration of tested plant extracts and the ordinate represented the average percent of scavenging capacity from three separate tests.

3. Results

3.1. *Optimization of sample extraction*

The compounds produced more complete peaks but interference peaks were small and the peak areas were larger in chromatograms when the samples were extracted with ultrasound than with reflux. The compounds produced more complete peaks but interference peaks were small and the peak areas were larger in chromatograms when the samples were extracted using methanol as a solvent than when using 95% ethanol as a solvent. Optimal extraction was achieved with ultrasound and methanol as a solvent.

3.2. *Comparison of compounds in three Rubus species extracts*

Figure 1A is the chromatogram for standard 2,6-dimethoxym -4-hydroxyphenol-1-*O*-β-D-glucopyranoside. Figure 1B is the chromatogram for compounds from the *R. amabilis* extract. Figure 1C is chromatogram for 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside in the *R. amabilis* extract. Figure 2A is the chromatogram for standard procyanidin B_4. Figure 2C is the chromatogram for procyanidin B_4 in the *R. amabilis* extract. Figure 3A is the chromatogram for standard isovitexin-7-*O*-glucoside. Figure 3C is chromatogram for isovitexin-7-*O*-glucoside in the *R. amabilis* extract. Peaks 1, 2, and 3 were identified by adding peaks for standard 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside, procyanidin B_4, and isovitexin-7-*O*-glucoside to peaks for the *R. amabilis* extract and then comparing Figures 1B and 1C, Figures 2B and 2C, and Figures 3B and 3C. The peak area for compound 1 in Figure 1C is larger than that in Figure 1B, the peak area for compound 2 in Figure 2C is larger than that in Figure 2B, and the peak area for compound 3 in Figure 3C is larger than that in Figure 3B. In contrast, the peaks for compounds 1, 2, and 3 are that were clearly produced by 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside, procyanidin B_4, and isovitexin-7-*O*-glucoside. Figure 4B is the chromatogram for compounds from the *R. niveus Thunb.* extract while Figure 4C is the chromatogram

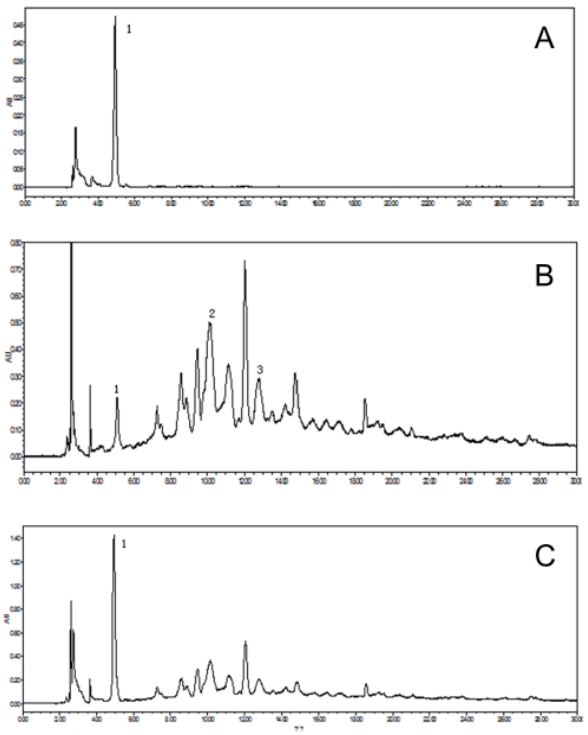

Figure 1. Chromatogram of standard 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside (A), *R. amabilis* extract (B), and *R. amabilis* extract with 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside (C).

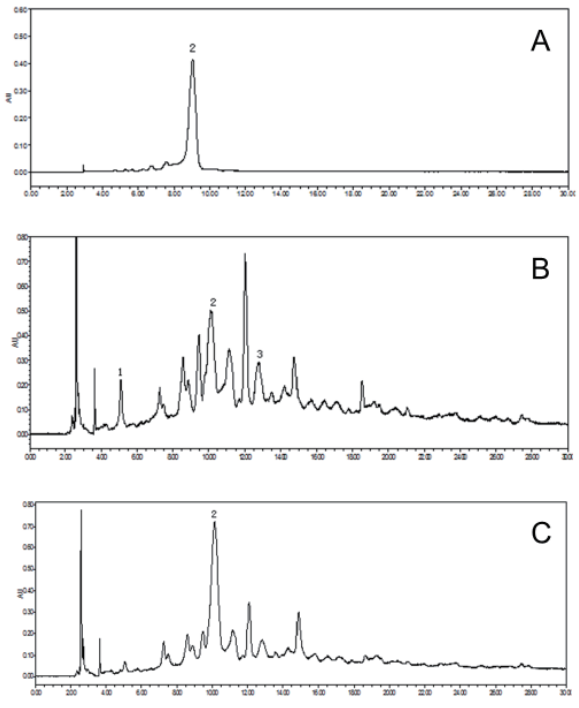

Figure 2. Chromatogram of standard procyanidin B_4 (A), *R. amabilis* extract (B), and *R. amabilis* extract with procyanidin B_4 (C).

for compounds from the *R. sachalinensis* extract. In comparison to the *R. amabilis* extract, these two *Rubus* species contained 2,6-dimethoxy-4-hydroxyphenol-1-

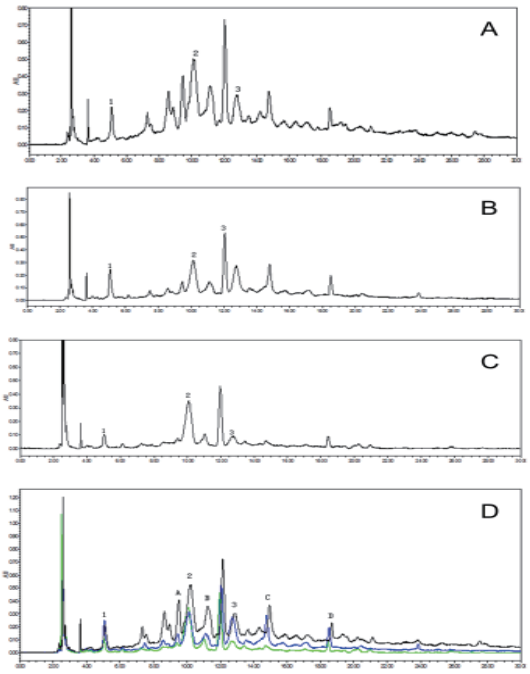

Figure 3. Chromatogram of standard isovitexin-7-*O*-glucoside (A), *R. amabilis* extract (B), and *R. amabilis* extract with isovitexin-7-*O*-glucoside (C).

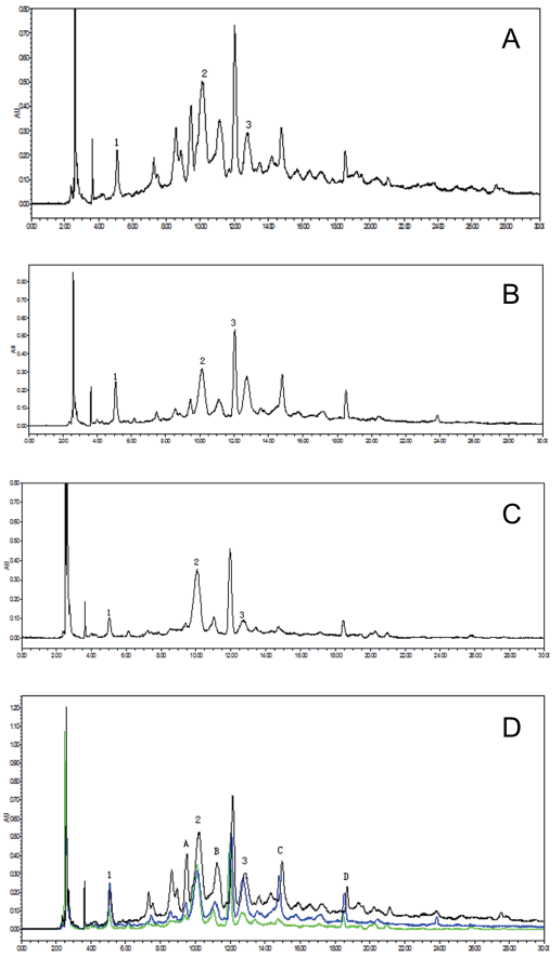

Figure 4. Chromatogram of *R. amabilis* extract (A), *R. niveus Thunb.* extract (B), and *R. sachalinensis* extract (C). Overlapping chromatograms for (A), (B), and (C) are shown in (D).

O-β-D-glucopyranoside with the same peak shape and same migration time. The three *Rubus* species contain procyanidin B_4 and isovitexin-7-*O*-glucoside with the same peak shapes and migration times. Many other similar peaks, such as peaks A, B, C, and D, are also present in Figure 4D.

3.3. *Antioxidant activity*

The antioxidant capacity of the extracts of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* was evaluated by determining the radical scavenging capacity with respect to DPPH (IC_{50} value) (Figure 5; Tables 2 and 3).

Extracts of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* were tested for their free radical scavenging of DPPH, and their IC_{50} values are shown in Table 2. Ascorbic acid, which was used as

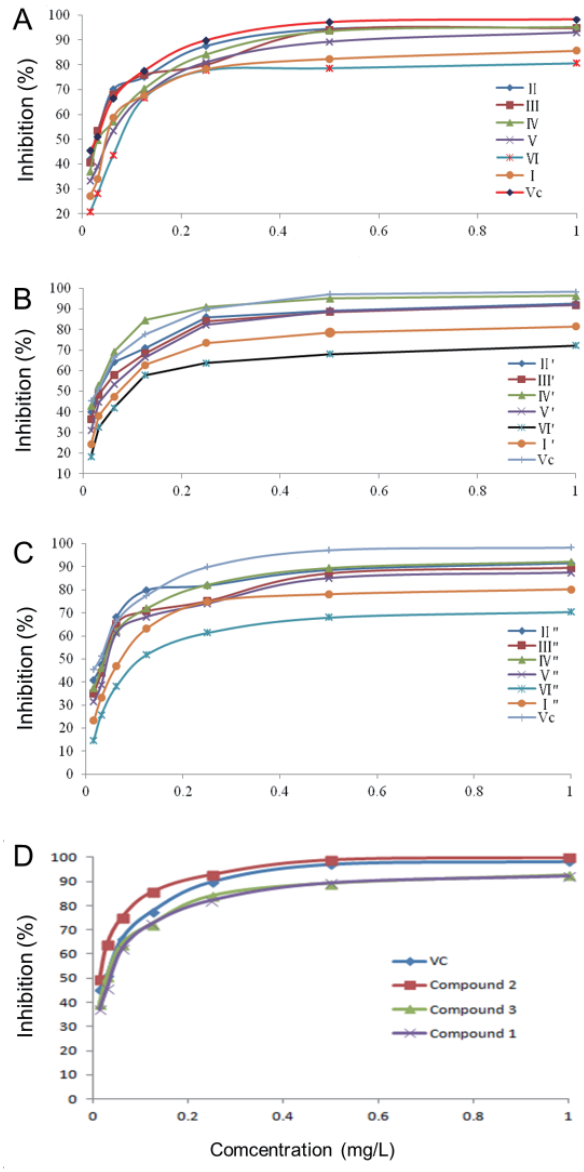

Figure 5. DPPH radical scavenging activity of *R. amabilis* I-VI and Vc (A), *R. niveus Thunb.* I′-VI′ and Vc (B), *R. sachalinensis* I″-VI″ and Vc (C), and compounds 1, 2, 3, and Vc (D).

Table 2. Radical scavenging activity of three *Rubus* species extracts

Sample	IC_{50} (mg/L)	Sample	IC_{50} (mg/L)
Vc	0.020	Vc	0.020
R. amabilis I	0.056	*R. niveus Thunb.* IV'	0.022
R. amabilis II	0.021	*R. niveus Thunb.* V'	0.046
R. amabilis III	0.025	*R. niveus Thunb.* VI'	0.108
R. amabilis IV	0.034	*R. sachalinensis* I"	0.077
R. amabilis V	0.048	*R. sachalinensis* II"	0.024
R. amabilis VI	0.083	*R. sachalinensis* III"	0.036
R. niveus Thunb. I'	0.069	*R. sachalinensis* IV"	0.033
R. niveus Thunb. II'	0.028	*R. sachalinensis* V"	0.047
R. niveus Thunb. III'	0.036	*R. sachalinensis* VI"	0.152

Note: I, I′, and I″ indicate samples extracted from 70% ethanol. II,II′, and II″ indicate samples extracted from petroleum ether. III, III′, and III″ indicate samples extracted from chloroform. IV, IV′, and IV″ indicate samples extracted from ethyl acetate. V, V, and V″ indicate samples extracted from n-butanol. VI, VI′, and VI″ indicate samples extracted from water.

Table 3. Radical scavenging activity of compounds 1-3 and Vc

Sample	IC_{50} (mg/L)
Vc	0.020
Compound 1	0.033
Compound 2	0.010
Compound 3	0.027

a positive control, had an IC_{50} value of 0.020 mg/L. All three *Rubus* species extracts had DPPH radical scavenging activity. In different solvents, *R. amabilis* extracts displayed antioxidant activity in the order of II>III>IV>V>I>VI. In different solvents, *R. niveus Thunb*. extracts displayed antioxidant activity in the order of IV'>II'>III'>V'>I'>VI'. In different solvents, *R. sachalinensis* extracts displayed antioxidant activity in the order of II">III">IV">V">I">VI". The petroleum ether, chloroform, and ethyl acetate extracts of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* had significantly less scavenging of DPPH. The aqueous extracts of the three *Rubus* species had less scavenging of DPPH in comparison to non-polar solvents. The three standards had antioxidant activity in the order of compound 2 > compound 3 > compound 1.

4. Discussion

Reflux extraction has conventionally been used to extract active compounds from plant samples. However, active compounds are readily lost due to evaporation, reaction, or oxidation during the long process of extraction. There has been considerable interest in the use of ultrasound to improve extraction from plant samples. Ultrasound-assisted extraction is one of the most inexpensive, simple, and efficient techniques (*11-13*) for extraction, and it can increase the yield of extracted components, reduce extraction time, and offer a high reprocessing throughput. The current study found that ultrasound-assisted extraction was superior to reflux extraction. Compounds 1 and 3 are glycosides and compound 2 is a biflavone. All three readily dissolve in a polar solvent.

Peaks 1, 2, and 3, which were produced by 2,6-dimethoxy-4- hydroxyphenol-1-*O*-β-D-glucopyranoside (compound 1), procyanidinB_4 (compound 2), and isovitexin-7-*O*-glucoside (compound 3), can be compared to peaks produced by *R. amabilis*, as shown in Figures 1, 4, and 6, in terms of the peak shape and the migration time. Comparison of Figure 2 to Figures 1, 4, and 6 does not indicate whether peaks 1, 2, and 3 were produced by 2,6-dimethoxy-4-hydroxyphenol-1-*O*-β-D-glucopyranoside, procyanidin B_4, or isovitexin-7-*O*-glucoside. However, peaks 1, 2, and 3 can be identified by adding peaks for standard compounds 1, 2, and 3 to peaks for the *R. amabilis* extract. Compounds 1, 2, and 3 are found in all three *Rubus* herbs extracts, but so are other compounds, as evident from peaks A, B, C, and D. According to the chromatograms, the three *Rubus* extracts contain 7 compounds. In conclusion, different regions substitute *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* for one another as a key ingredient in Manubzhithang.

Three standard compounds and three *Rubus* species extracts were compared in terms of their antioxidant activity *in vitro*. The antioxidant activity of the three *Rubus* species extracts differed with different solvents. The three *Rubus* species extracts had similar levels of antioxidant activity *in vitro*. For instance, the petroleum ether, chloroform, and ethyl acetate extracts of three *Rubus* species displayed marked antioxidant activity *in vitro* while the n-butanol and aqueous extracts displayed weak antioxidant activity *in vitro*.

The petroleum ether, chloroform, and ethyl acetate extracts of *R. amabilis*, *R. niveus Thunb.*, and *R. sachalinensis* had significantly less scavenging of DPPH in comparison to polar solvents. However, a study of *R. amabilis* separated compounds 2 and 3 from n-butanol and compound 1 from ethyl acetate (*14*). This finding suggests that the petroleum ether, chloroform, and ethyl acetate extracts contain more flavonoids such as quercetin, 8-dihydroxy-3,7-dimethoxyxanthone, 1-hydroxy -3,7,8-trimeth-oxyxanthone, and 8-dihydroxy-3,5-dimethoxyxanthone (*14*). These substances display

exceptional antioxidant properties *in vitro*. Compounds 2 and 3 had superior antioxidant properties *in vitro* when extracted with a highly polar solvent. However, the three *Rubus* species extracts could contain other compounds that do not possess radical scavenging activity when extracted with a polar solvent like n-butanol and water. The current results indicated that extracts of the three *Rubus* species had almost the same level of scavenging of DPPH despite extraction with different solvents.

The current study is the first to compare compounds of three *Rubus* species and their antioxidant activity. Findings indicated the rationality of substituting the three *Rubus* species for one another as a key ingredient in the traditional Tibetan medicine Manubzhithang.

Acknowledgements

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References

1. Tadesse S, Asres K, Veeresham C. Antioxidant activities of three *Rubus* species growing in Ethiopia. Ethiop Pharm J. 2007; 25:103-110.
2. Xiaochuan C, Qixiu Z, Zhong J. Pregnane glycoside, lignan glycosides, triterpene glycosyl ester and flavonoid glycosides from *Rubus* amabilis. Planta Med. 2001; 67:270-273.
3. Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Oxidative stress and stress activated signaling pathways: A unifying hypothesis of type 2 diabetes. Endocr Rev. 2002; 23:599-622.
4. McCune LM, Johns T. Antioxidant activity relates to plant part, life form and growing condition in some diabetes remedies. J Ethnopharmacol. 2007; 112:461-469.
5. Rahimi R, Nikfar S, Larijani B, Abdollahi M. A review on the role of antioxidants in the management of diabetes and its complications. Biomed Pharmacother. 2005; 59:365- 373.
6. Yau MH, Che CT, Liang SM, Kong YC, Fong WP. An aqueous extract of *Rubus* chingii fruits protects primary rat hepatocytes against tert-butyl hydroperoxide induced oxidative stress. Life Sci. 2002; 72:329-338.
7. Jiangsu College of New Medicine. A Dictionary of Traditional Chinese Medicine. Shanghai: Shanghai People's Publishing House, 1977:1880.
8. George BP, Parimelazhagan T, Saravanan S, Chandran R. Anti-inflammatory, analgesic and antipyretic properties of *Rubus* niveus Thunb. root acetone extract. Pharmacologia. 2013; 4:228-235.
9. George BP, Parimelazhagan T, Sajeesh T, Saravanan S. Antitumor and wound healing properties of *Rubus* niveus Thunb. root. J Environ Pathol Toxicol Oncol. 2014; 33:145-158.
10. Yutu YDGB. Si Bu Yi Dian. Tibet People Press, Tibet, China, 2000; 572-576. (in Chinese)
11. Chen XP, Wang WX, Li SB, Xue JL, FanLJ, Sheng ZJ, Chen G. Optimization of ultrasound-assisted extraction of Lingzhi polysaccharides using response surface methodology and its inhibitory effect on cervical cancer cells. Carbohydr Polym. 2010; 80:944-948.
12. Yan YL, Yu CH, Chen J, Li XX, Wang W, Li SQ. Ultrasonic-assisted extraction optimized by response surface methodology, chemical composition and antioxidant activity of polysaccharides from Tremella mesenterica. Carbohydr Polym. 2011; 83:217-224.
13. Zhong K, Wang Q. Optimization of ultrasonic extraction of polysaccharides from dried longan pulp using response surface methodology. Carbohydr Polym. 2010; 80:19-25.
14. Rezeng CD, Limao CR, LIU B. Chemical constituents of Tibetan medicine kandrakari (I). Chin J Chin material Med. 2014, 39:1168-1172.

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Original Article

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Preparation and characterization of lidocaine rice gel for oral application

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Summary

The objective of the present study was to prepare buccal anesthetic gels using rice as gelling agent. Rice grains of four rice varieties, Jasmine (JM), Saohai (SH), Homnil (HN), and Doisket (DS) were chemically modified. Buccal rice gels, containing lidocaine hydrochloride as local anesthetic drug were formulated using the respective modified rice varieties. The gels were evaluated for outer appearance, pH, color, gel strength, foaming property, adhesion, *in vitro* drug release and *in vivo* efficacy. It was found that the developed rice gels possessed good texture. Rice varieties showed influence on gel strength, color, turbidity, adhesive property, release property, and anesthetic efficacy. JM gel showed the lowest turbidity with light transmission of 86.76 ± 1.18% whereas SH gel showed the highest gel strength of 208.78 ± 10.42 g/cm^2. Lidocaine hydrochloride can cause a decrease in pH and adhesive property but an increase in turbidity of the gels. *In vitro* drug release profile within 60 min of lidocaine SH gel and lidocaine HN gel showed that lidocaine could be better released from SH gel. Evaluation of *in vivo* anesthetic efficacy in 100 normal volunteers indicates that both lidocaine rice gels have high efficacy but different levels. Lidocaine SH gel possesses faster onset of duration and longer duration of action than lidocaine HN gel.

Keywords: Rice gel, local anesthetic, mucoadhesive, drug release, lidocaine

1. Introduction

Many dental treatment procedures *e.g*. tooth extraction, scaling and root planing cause severe pain to the patients. These procedures therefore need an anesthetic drug to restrain the pain during the treatment. Anesthetic drug administration is generally done by injection. However, the first anesthetic injection also causes pain to the patients and makes them fear of dentistry (*1*). To reduce this pain, several methods have been used such as using a fine-gauge needle and gently pierce the needle to the target area (*2*), using a slower rate of injection in order to reduce the tissue tension (3,4), adjusting the pH or buffering the anesthetic solution in order to reduce the burning pain from the acidity of its salt solution (*5,6*), and using topical anesthetic application prior to injection (*7*). Among these methods, topical application of local anesthetic dosage form to the injection area prior to insert the needle is the most effective to overcome the pain at needle puncture. Many dentists therefore prefer to apply topical anesthetic dosage form before oral treatment with injection (*8,9*). Lidocaine has been used for local anesthesia via many routes of administration such as intradermal injection and topical application. Its anesthetic activity is due to the neuronal voltage-gated Na^+ channel blocking. This activity leads to failure in the generation or propagation of peripheral nerve action potential (*10*).

There are many dosage forms that can be applied topically in oral cavity such as disks (*11*), tablets (*12*), patches (*13*), films (*14*), ointments (*15*), and gels (*16*). Among these formulations, buccal gels are the most preferable in terms of patient compliance, comfort, and easy dispersion throughout the mucosa. Moreover,

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buccal gels show prolonging residence time at the site of drug absorption and high mucoadhesive on the absorption surface (*17*).

Gels are made of various synthetic polymers such as polyvinylalcohol, polyvinylpyrrolidone, and poly (acrylic acid). However, using chemical synthetic polymers may cause serious environmental problems. Using polymers from natural resources therefore are of better options. Recently, we reported that the gels derived from rice grain powder of some rice strains possess high mucoadhesive (*18*). In the present study, lidocaine rice gels were prepared and their characteristics and efficacy were compared.

2. Materials and Methods

2.1. *Rice materials and chemicals*

Milled rice grains of different rice varieties in Thailand, Jasmine (JM), Saohai (SH), Homnil (HN), and Doisket (DS) were used. JM and SH are white rice grains where HN and DS are reddish purple rice grains as shown in Figure 1. All rice grains used were harvested during July – September, 2014. 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). Lidocaine hydrochloride (99.9% purity) was from Gufic Biosciences (Mumbai, India). All other chemicals and solvents were of AR grade or the highest grade available.

2.2. *Preparation of lidocaine rice gel*

The rice grains of each variety were modified and used as gelling agent. The rice modification procedure was done according to the method reported by Okonogi *et al*. (*19*) with some modification. Briefly, the raw rice was firstly added into sodium hydroxide methanol solution. After that, monochloroacetic acid solution was added and refluxed at 60°C for 3 h. The solid granules obtained were washed with ethanol until the silver nitrate test for chloride of the filtrate was negative. The dried solid modified rice was pulverized and the fine powder that passed an 80-mesh sieve was used for gel base preparation. Rice gel base of each rice variety was prepared using hydration method. The dispersions of the modified rice powder and water were heated to 90°C in a closed chamber for 2 h and gently stirred to obtain homogenous gels without air bubble. Lidocaine hydrochloride was exactly weighed and gradually levigated into the rice gel bases until the transparent gels of 2% lidocaine were obtained.

2.3. *pH and turbidity study of the gels*

The pH of the rice gel bases in comparison with lidocaine rice gels was measured using a pH meter with an electrode probe for semisolid samples. The turbidity of the gels was measured using a spectrophotometer (JASCO Corporation, Tokyo, Japan) at 620 nm against distilled water. Each turbidity measurement was carried out in triplicate.

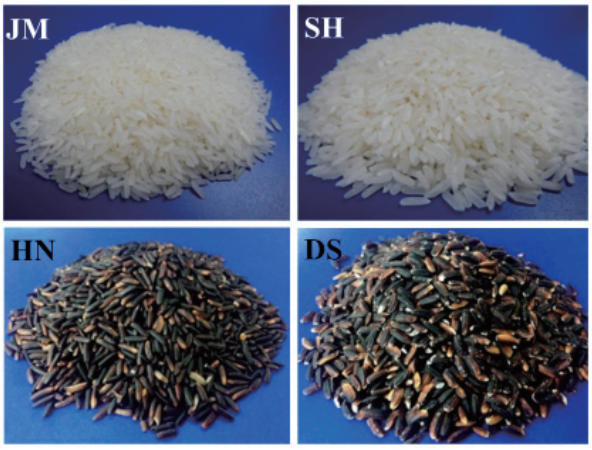

Figure 1. Outer appearance of four rice grains.

2.4. *Color measurement of the gels*

The color of lidocaine rice gels was determined using a chroma meter (Konica Minolta Optics Inc, Osaka, Japan) and Spectra-Match software, sets to lightness (L^*), redness (a^*), and yellowness (b^*) mode. The method of determination was according to the previous report (*20*). The samples were filled in a glass sample cup. Determination was performed on cross-sections of the sample. Ten determinations were performed on each gel sample. The white calibration plate was used to standardize the equipment.

2.5. *Determination of gel strength*

The gel strength of lidocaine rice gels was investigated by a method described by Zhou and Regenstein (*21*) using a texture analyzer (Stable Micro Systems Ltd., Godalming, United Kingdom). The gels were filled in a cylindrical cup (30 mm diameter × 15 mm height) and kept at 6 ± 1°C for 16-18 h. The measurement was performed using a 12.7-mm diameter plunger. The test mode was compression and the penetration speed was constant at 2.00 mm/sec and the penetration depth applied was 4 mm. The gel strength is a maximum force required in penetration. Determination was made in triplicate.

2.6. *Foam forming of the gels*

Foam forming of rice gel bases in comparison with lidocaine rice gels was measured by the method previously described (*22*) with some modification.

Briefly, the 1 mL gel sample was filled into a 10 mL graduated cylinder. The 4 mL of water was added. The cylinder was closed tightly and was vigorously shaked for 20 times. The foam volumes generated and floated over the top layer of the liquid after stop shaking (0 min) and the foam volume left after stop shaking for 15 min were recorded. Foaming properties were calculated as follow.

$$\text{Foam forming ability} = \frac{\text{Volume of foam at 0 min}}{\text{Initial volume of gel solution}}$$

$$\text{Foam stability} = \frac{\text{Volume of foam at 15 min}}{\text{Volume of foam at 0 min}}$$

2.7. *Adhesive property test of the gels*

2.7.1. *Thumb test*

This method was performed as the previous report (*23*), with some modification. Briefly, the gel sample was placed between the tips of thumb finger and middle finger and kept as such for exact time of 1 min. The qualitative adhesiveness was measured by the difficulty in separating the fingertips.

2.7.2. *Tack determination*

The tack is the ability of a gel sample to bond under conditions of light contact pressure and a short contact time. In the present study, the tack of the adhesive surface contact of the gel was measured by the rolling ball tack test. The exact amount of gel sample was applied thoroughly on the smooth surface plate with a width of 20 mm and a length of 100 mm. This plate was laid horizontally next to the inclined plate. A 15-mm diameter glass ball was released from the top of the inclined plate (angle 30°) with a running length of 200 mm and let it run on the adhesive plate until stopped by adhesive power of the gel. The length of the adhesive plate that the ball can run from the beginning of the plate to the stop point was recorded as the tack value.

2.8. *In vitro drug release property of the gels*

The *in vitro* release study was performed using dialysis bag with a molecular weight (MW) cut-off at 12,000 daltons (Cellu Sep® T4 regenerated cellulose tubular membrane, Membrane Filtration Products, Inc., TX, USA). The receptor compartment had a capacity of 50 mL. The dialysis bag was degassed and saturated for 30 min in receptor medium (phosphate buffer pH 7.4) before starting the experiment. The gel sample of 1 g was placed in the hydrated dialysis bag with the aid of a syringe and checked for air bubbles. The dialysis bag was tightly closed and then immersed into the medium. The receptor medium was maintained at 37 ± 1°C under constant stirring of 100 rpm. To characterize the drug release, samples were collected after 5, 10, 15, 20, 30, 40, 50 and 60 min. After sampling, the volume collected was replaced with fresh receptor medium. The amount of lidocaine released was determined by HPLC with UV detection at 230 nm. Elution was carried out at room temperature with a mobile phase consisting of phosphate buffer pH 8 (60%) and acetonitrile (40%); the injecting volume was 20 µL. The flow rate was 1.0 mL/min. In these conditions the retention time of lidocaine is 15.0 min. A calibration curve was prepared using lidocaine solution at concentrations ranging from 1 to 10 µmol/mL. In this range the method gave a linear response (r^2 = 0.9998).

2.9. *In vivo anesthetic activity of the gels*

Subjects for this study were recruited from normal volunteers of Chiang Mai University (CMU). This study was approved by the Human Experimentation Committee, Faculty of Dentistry, CMU. The anesthetics that were used in the study were two selected lidocaine rice gels. Subjects were randomly assigned to one of two groups: Each group (n = 50) received a completely blind separated lidocaine rice gel sample. An aliquot of 0.1 mL gel was placed on the tip of the tongue. The tongue was scratched using the fine gauge needle to determine the onset and duration of the anesthetic action.

2.10. *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 a SPSS software version 11.

3. Results and Discussion

3.1. *Preparation of lidocaine rice gels*

Previous reports demonstrated that rice grain composed mainly of starch (*18*). The chemical modification of rice starch under etherification used in this study could produce carboxymethyl starch. This reaction is to substitute carboxymethyl groups (CH_2COO-), which are negatively charged, for hydroxyl groups (–OH) in starch molecules (Volkert *et al*., 2004). The raw rice powders of white rice grains (JM and SH) and color rice grains (HN and DS) have slightly different in color but their modified rice powders showed similar appearance as shown in Figure 2. It is noted that the color of the modified rice powders derived from the color rice varieties was changed to almost white. The modified rice powders obtained showed good property

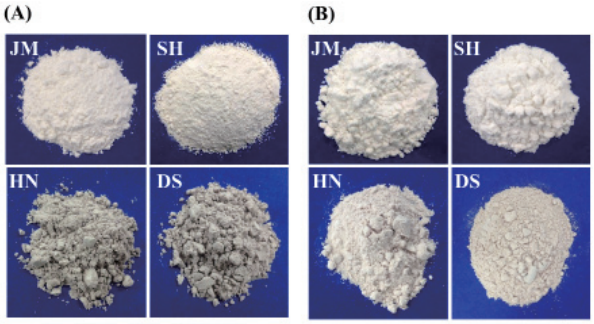

Figure 2. Outer appearance of raw rice powders (A) and modified rice powders (B).

Table 1. pH of rice gels

Rice gels	pH	
	Gel bases	Lidocaine gels
JM	7.0	6.5
SH	9.0	6.6
HN	7.8	5.9
DS	7.5	5.8

for forming gel base under simple hydration method. All rice gel bases showed good compatibility to lidocaine hydrochloride. Lidocaine rice gels obtained from these four different modified rice gel bases were further investigated for their characteristics.

3.2. *pH and turbidity of the gels*

Four rice gel bases and lidocaine loaded rice gels were compared. The pH of the gel bases was in the range of 7.0-9.0. After incorporating with lidocaine hydrochloride, the pH of the gels was slightly decreased as seen in Table 1. Lidocaine is a weak base having pKa of 7.92 at 25°C. However, the aqueous solution of its salt form with strong acid like hydrochloride form shows the weak acid to litmus. This effect therefore influenced the pH of lidocaine rice gels to be lower than their respective gel bases. Lidocaine cannot dissolve easily in water but in the form of hydrochloride, the drug solubility in aqueous system like rice hydrogel can be increased. Therefore, there was no precipitation of the drug observed in the gels. However, the gels obtained were not completely transparent. The turbidity of the gels then was measured by means of light transmission. It was found that there was a significant difference in gel turbidity among the gels obtained from different rice varieties as shown in Table 2. The highest transmission of light at 620 nm which indicated the lowest gel turbidity was found in JM gel. Lidocaine rice gels showed slightly higher turbidity than their respective gel bases. It was reported that the addition of ions might cause the increase of gel turbidity due to an increase of the aggregation of helices to form a three dimensional network (*24*). However, it was reported that the addition of small amount of calcium

Table 2. Transmission of rice gels at 620 nm

Rice gels	Transmission of light (%)	
	Gel bases	Lidocaine gels
JM	86.76 ± 1.18	58.67 ± 0.90
SH	79.19 ± 0.76	40.63 ± 0.41
HN	66.51 ± 1.07	55.27 ± 0.59
DS	72.08 ± 1.27	43.58 ± 0.12

Table 3. Color of lidocaine rice gels measured by light reflection method

Rice gels	L^*	a^*	b^*
JM	30.02 ± 0.92	– 0.23 ± 0.03	1.25 ± 0.16
SH	32.76 ± 0.61	– 0.38 ± 0.04	0.27 ± 0.03
HN	24.62 ± 0.28	3.36 ± 0.26	4.17 ± 0.63
DS	24.24 ± 0.33	3.36 ± 0.78	6.36 ± 0.47

could prevent aggregation formation of gelatin (*25*). It was also reported that the turbidity data did not reveal whether turbidity was caused by either the formation of more or larger aggregates, or both. However, in most cases, gel turbidity is caused by the scattering of light by particles entrapped inside the gel matrix (*26*). It was reported that transparent gels consisted of a molecularly homogeneous network, whereas nontransparent gels consisted of colloid particles or aggregates which larger than one quarter of the wavelength of light above 150 nm (*27*). In the present study, the addition of lidocaine hydrochloride to the rice gel bases showed a slight increase of gel turbidity. It is considered that there might be the formation of some tiny aggregates of drug molecules inside the gel network.

3.3. *Color of lidocaine rice gels*

Color of the gel is important factor in terms of general appearance and consumer acceptance. The outer appearance of lidocaine gels obtained from four different rice varieties was similar but different in color. Visual observation demonstrated that the gels derived from white rice strains were slightly white where those obtained from the color rice were slightly red purple in color. Due to different turbidity, color of the gels was measured by light reflection method using a chroma meter. This measurement reflects three color parameters; L^*, a^*, and b^*. The parameter L^* refers to the lightness of the samples, and ranges from black ($L = 0$) to white ($L = 100$). A negative value of parameter a^* indicates green, while a positive one indicates red–purple color. Positive value of parameter b^* indicates yellow while negative value indicates blue color. The results of color measurement of the gels therefore are shown as the values of L^*, a^*, and b^*. As shown in Table 3, it is found that between the two white rice gels; the color of SH gel was whiter and less yellow than JM gel. Both color rice gels showed less whiteness than white

rice gels where DS showed higher yellow than HN. The color of the rice gels observed by visualization is found to be the blend color of L^*, a^*, and b^*.

3.4. *Gel strength*

Gel strength is one of the important properties of pharmaceutical gels. Previous report showed that the gel strength of various starches depended on the type and concentration of the starches (*28*). Moreover, it was reported that the gel strength of agar extracted from the same genus but different species was significantly different (*29*). In the present study, rice gels derived from the same plant species of rice (*Oryza sativa* Linn.) but different varieties was studied. It is found that the gel strength of white rice is higher than that of color rice. The highest gel strength was obtained from lidocaine SH gel with the gel strength value of 208.78 ± 10.42 g/cm2 followed closely by JM gel with the value of 181.13 ± 13.92 g/cm^2. HN and DS gels demonstrated significantly lower gel strength values of 154.47 ± 6.11 g/cm^2 and 157.89 ± 8.49 g/cm^2, respectively. This result reveals that the plant variety also plays an important role on the gel strength.

3.5. *Foam properties of the rice gels*

Foam is a dispersion of gas bubbles in the liquid or semisolid systems. Foam formation can be easily formed during stirring certain systems containing component having activity to decrease surface tension. A desirable good appearance pharmaceutical gel should not contain any foams or air bubbles. Therefore, it is essential to investigate the possibility of foam forming and foam stability obtained by rice gels in order to avoid those undesirable foams in the formulated gels. The results showed that foam formation could be occurred in rice gel bases but in tiny amount. The ability of foam forming is depended on the rice variety as seen in Figure 3. Gel bases obtained from color rice varieties showed higher foam forming ability than those obtained from white rice varieties. The highest foam forming ability was found in HN gel with 0.1 times of the initial gel solution. Interestingly, after adding lidocaine hydrochloride in the gel bases, the foam formation was dramatically decreased. The foams of rice gels showed low stability. After 15 min of stop shaking, the foams of all gel bases decreased rapidly, particularly in the gels containing lidocaine hydrochloride as seen in Figure 4. Previous report demonstrated that adding some ions or compounds could interrupt foam forming in different mechanisms (*30,31*). The results of the present study showed that adding of lidocaine hydrochloride to the rice gel bases can prevent the formation of undesirable foam in the obtained gels.

3.6. *Adhesive property of the gels*

The adhesive property is essential for buccal drug delivery (*32*). The adhesive property of lidocaine rice gels in this study was investigated by thumb test and tack determination. The thumb test is a simple test method. The results from thumb test can roughly identify adhesiveness of the test samples. Although the thumb test may not be conclusive, it provides useful information on mucoadhesive potential. The difficulty of pulling the thumb from the adhesive gels is a function of the pressure and the contact time. Like mucin, the skin has many hydroxyl groups. It is likely that any mucoadhesive system is adhesive to fingers, since most mucoadhesives are nonspecific and not mucin specific. The results of this study revealed that all rice gel bases as well as the lidocaine rice gel bases obtained possessed good adhesive property (data not shown). Further investigation of more precisely adhesive property was done using tack test. The result was expressed as the tack value which represented the length of the adhesive plate (covered with the gel sample) that the ball could run from the beginning of the adhesive plate applied with the rice gel sample to the stop point. Therefore, the lower tack value indicates the higher adhesive property of the gel. The results are shown in Table 4. From this result, it is seen that the gel bases of the white rice possessed lower adhesive strength than that of the color rice. Lidocaine

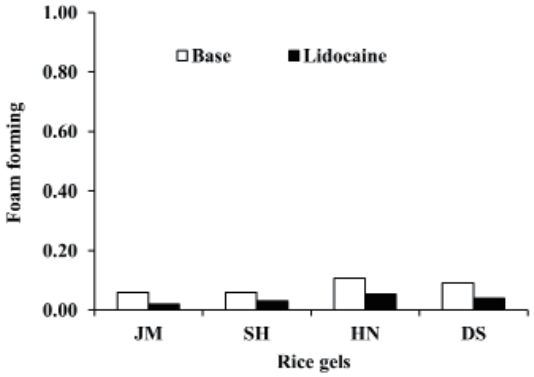

Figure 3. Foam forming ability of rice gels.

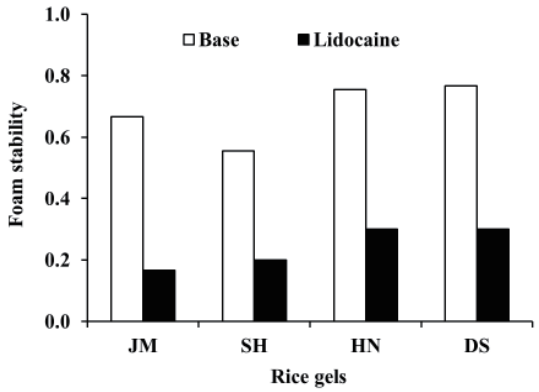

Figure 4. Foam stability of rice gels.

Table 4. Tack value of the rice gels

Rice gels	Tack value (cm)	
	Gel bases	Lidocaine gels
JM	5.20 ± 0.17	6.39 ± 0.22
SH	4.95 ± 0.20	5.62 ± 0.23
HN	3.87 ± 0.15	5.01 ± 0.30
DS	4.38 ± 0.13	6.28 ± 0.13

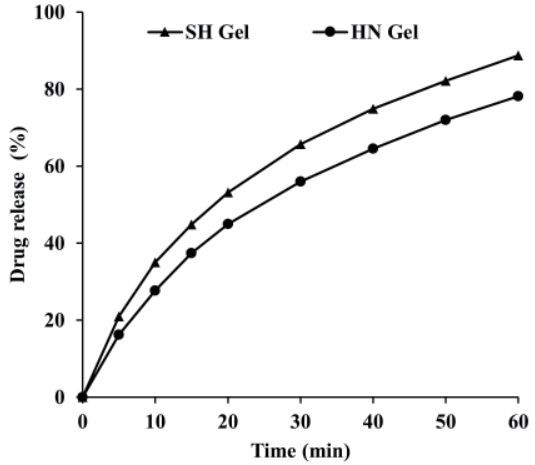

Figure 5. *In vitro* release profiles of lidocaine rice gels.

hydrochloride caused slightly higher tack value indicating slightly decrease in adhesive property of the gels.

3.7. *In vitro drug release property and in vivo efficacy of lidocaine rice gels*

In this experiment the white rice SH gel and the color HN gel were selected and their *in vitro* drug release properties as well as *in vivo* anesthetic efficacy were compared. The *in vitro* drug release tests were carried out in pH 7.4 buffer. It was observed that the release is accompanied by the dissolving of the gels. However, only the drug molecules could diffuse through the used definite MW cut-off dialysis membrane. The release profile of the two gel samples is presented in Figure 5. It is shown that SH rice gel possesses higher drug release property than HN gel. Further study in human volunteers was done in order to compare the anesthetic activity of both gels. It was found that both lidocaine rice gels possess anesthetic efficacy but in different level. Figure 6 demonstrates the results as onset and duration of action of both comparative gels. Lidocaine SH rice gel showed faster onset of action (23.96 ± 11.51 min) and longer duration of action (337.99 ± 68.55 min) than lidocaine HN gel. The onset of action of these gels is in correspondence with the results in *in vitro* release study. Lidocaine could be released from SH gel faster than from HN gel, therefore it showed faster onset of action than lidocaine HN gel. According to the difference in duration of action between these two gels,

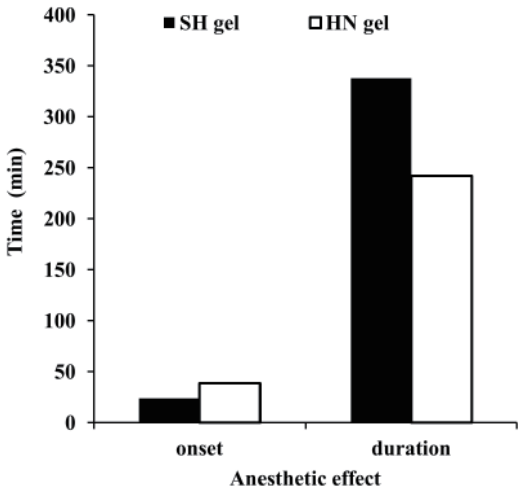

Figure 6. Onset and duration of action of lidocaine rice gels.

it is considered that there might be some interaction between the drug and the components existing in only the SH gel which could be resulted as a sustain release activity.

4. Conclusion

Lidocaine rice gel was developed. The effects of rice varieties on characteristics of lidocaine rice gels were investigated. The color of rice gels obtained was in accordance with the color of their respective rice varieties. The rice gel showed less ability of foam forming and the foams formed were less stability. White rice varieties yield the gels with higher gel strength than color rice varieties. Gel bases of white rice varieties have higher adhesive property than that of color varieties. pH of the gels obtained from both white rice and color rice varieties are similar and nearly 7.0. Incorporating the rice gel bases with lidocaine hydrochloride can cause decrease in pH and adhesive property of the gels. However, lidocaine hydrochloride increases turbidity of the gels. The developed lidocaine rice gels possess anesthetic efficacy. Lidocaine gel of white rice variety possesses higher ability of drug release, faster onset of action and longer duration of action than that of color rice variety.

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References

1. Kleinknecht RA, Klepac RK, Alexander LD. Origins and characteristics of fear of dentistry. J Am Dent Assoc. 1973; 86:842-848.
2. Watts AC, McEachan J. The use of a fine-gauge needle to reduce pain in open carpal tunnel decompression: A randomized controlled trial. J Hand Surg Br. 2005; 30:615-617.
3. Arndt KA, Burton C, Noe JM. Minimizing the pain of local anesthesia. Plast Reconstr Surg. 1983; 72:676-679.
4. Scarfone RJ, Jasani M, Gracely EJ. Pain of local anesthetics: Rate of administration and buffering. Ann Emerg Med. 1998; 31:36-40.
5. Hanna MN, Elhassan A, Veloso PM, Lesley M, Lissauer J, Richman JM, Wu CL. Efficacy of bicarbonate in decreasing pain on intradermal injection of local anesthetics: A meta-analysis. Reg Anesth Pain Med. 2009; 34:122-125.
6. Cheney PR, Molzen G, Tandberg D. The effect of pH buffering on reducing the pain associated with subcutaneous infiltration of bupivicaine. Am J Emerg Med. 1991; 9:147-148.
7. Lawrence TM, Desai VV. Topical anaesthesia to reduce pain associated with carpal tunnel surgery. J Hand Surg Br. 2002; 27:462-464.
8. Hersh EV, Houpt MI, Cooper SA, Feldman RS, Wolff MS, Levin LM. Analgesic efficacy and safety of an intraoral lidocaine patch. J Am Dent Assoc. 1996; 127:1626-1634.
9. Svensson P, Petersen JK, Svensson H. Efficacy of a topical anesthetic on pain and unpleasantness during scaling of gingival pockets. Anesth Prog. 1994; 41:35-39.
10. Yanagidate F, Strichartz GR. Local anesthetics. Handb Exp Pharmacol. 2007; 177:95-127.
11. El-Samaligy MS, Yahia SA, Basalious EB. Formulation and evaluation of diclofenac sodium buccoadhesive discs. Int J Pharm. 2004; 286:27-39.
12. Onishi H, Yumoto K, Sakata O. Preparation and evaluation of ritodrine buccal tablets for rational therapeutic use. Int J Pharm. 2014; 468:207-213.
13. Shiledar RR, Tagalpallewar AA, Kokare CR. Formulation and *in vitro* evaluation of xanthan gum-based bilayered mucoadhesive buccal patches of zolmitriptan. Carbohyd Polym. 2014; 101:1234-1242.
14. Jones E, Ojewole E, Pillay V, Kumar P, Rambharose S, Govender T. Monolayered multipolymeric buccal films with drug and polymers of opposing solubility for ARV therapy: Physico-mechanical evaluation and molecular mechanics modelling. Int J Pharm. 2013; 455:197-212.
15. Petelin M, Pavlica Z, Bizimoska S, Sentjurc M. *In vivo* study of different ointments for drug delivery into oral mucosa by EPR oximetry. Int J Pharm. 2004; 270:83-91.
16. Rai VK, Yadav NP, Sinha P, Mishra N, Luqman S, Dwivedi H, Kymonil KM, Saraf SA. Development of cellulosic polymer based gel of novel ternary mixture of miconazole nitrate for buccal delivery. Carbohyd Polym. 2014; 103:126-133.
17. Harris D, Robinson JR. Drug delivery via the mucous membranes of the oral cavity. J Pharm Sci. 1992; 81:1-10.
18. Okonogi S, Kaewpinta A, Khongkhunthien S, Yotsawimonwat S. Effect of rice variety on the physicochemical properties of the modified rice powders and their derived mucoadhesive gels. Drug Discov Ther. 2015; 9:221-228.
19. Okonogi S, Khongkhunthien S, Jaturasitha S. Development of mucoadhesive buccal films from rice for pharmaceutical delivery systems. Drug Discov Ther. 2014; 8:262-267.
20. Pintado T, Ruiz-Capillas C, Jiménez-Colmenero F, Carmona P, Herrero AM. Oil-in-water emulsion gels stabilized with chia (*Salvia hispanica* L.) and cold gelling agents: Technological and infrared spectroscopic characterization. Food Chem. 2015; 185:470-478.
21. Zhou P, Regenstein JM. Optimization of extraction conditions for pollock skin gelatin. J Food Sci. 2004; 69:C393-C398.
22. Marinova KG, Basheva ES, Nenova B, Temelska M, Mirarefi AY, Campbell B, Ivanov IB. Physico-chemical factors controlling the foamability and foam stability of milk proteins: Sodium caseinate and whey protein concentrates. Food Hydrocolloids 2009; 23:1864-1876.
23. Sahu BP, Sharma HK, Das MK. Development and evaluation of a mucoadhesive nasal gel of felodipine prepared with mucoadhesive substance of *Dillenia indica* L. Asian J Pharm Sci. 2011; 5:175-187.
24. Lau MH, Tang J, Paulson AT. Texture profile and turbidity of gellan/gelatin mixed gels. Food Res Int. 2000; 33:665-671.
25. Lau MH, Tang J, Paulson AT. Effect of polymer ratio and calcium concentration on gelation properties of gellan/gelatin mixed gels. Food Res Int. 2001; 34:879-886.
26. Kitabatake N, Doi E, Kinekawa Y. Simple and rapid method for measuring turbidity in gels and sols from milk whey protein. J Food Sci. 1994; 59:769-772.
27. Paulsson M, Dejmek P, Vliet TV. Rheological properties of heat-induced beta-lactoglobulin gels. J Dairy Sci. 1990; 73:45-53.
28. Hamer WJ. An improved method for measurement of gel strength and data on starch gels. J Res Natl Bur Stand. 1947; 9:29-37.
29. Istinii S, Masao O, Kusunose H. Methods of analysis for agar, carrageenan and alginate in seaweed. Bull Mar Sci Fish–Kochi Univ. 1994; 14:49-55.
30. Kulkarni RD, Goddard ED, Kanner B. Mechanism of antifoaming action. J Colloid Interf Sci. 1977; 59:468-476.
31. Denkov ND, Marinova KG, Tcholakova SS. Mechanistic understanding of the modes of action of foam control agents. Adv Colloid Interf Sci. 2014; 206:57-67.
32. De Vries ME, Boddé HE, Verhoef JC, Junginger HE. Developments in buccal drug delivery. Crit Rev Ther Drug Carrier Syst. 1991; 8:271-303.

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Original Article

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High prevalence of *VKORC1*3* (G9041A) genetic polymorphism in north Indians: A study on patients with cardiac disorders on acenocoumarol

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Summary **Coumarin derivatives such as warfarin and acenocoumarol are used in various disorders such as deep venous thrombosis, pulmonary embolism, atrial fibrillation and artificial heart valves. They have improved prognosis of patients with thromboembolic disease. An individual's response to coumarins depends on several factors. The non-genetic factors include age, gender, body mass index, diet and interacting drugs. Among the genetic factors, the cytochrome P450 system and vitamin K epoxide reductase complex subunit 1 play a key role in drug metabolism. This was a prospective hospital based study in which allele and genotypic frequencies of *CYP2C9* gene polymorphisms; 430C>T and 1075A>C and *VKORC1* gene polymorphisms; 1639G>A, 9041G>A and 6009C>T in 106 alleles of north Indian patients with valve replacement on acenocoumarol were determined and their effect on acenocoumarol dosing was studied. To the best of our knowledge, this is first report of *VKORC1* 9041G>A and 6009C>T gene polymorphisms and their effect on acenocoumarol dosing from north India. In 53 patients with valve replacement on acenocoumarol with stable INR, the allele frequency of *CYP2C9*2* and *CYP2C9*3* gene polymorphisms was 0.05 and 0.17 respectively and that of *VKORC1 *2,*3* and **4* gene polymorphisms was 0.15, 0.72 and 0.11 respectively. The presence of *CYP2C9*3* or *VKORC1*2* gene polymorphism were associated with decrease in acenocoumarol dose requirements (p values 0.03 and 0.02 respectively).This study confirmed the association of lower mean weekly dosages of acenocoumarol in patients with *CYP2C9*3* and *VKORC1*2* gene polymorphisms. An unusually high frequency of 9041A polymorphism in *VKORC1* was found in study population.**

Keywords: *CYP2C9*, *VKORC1*, acenocoumarol, dosage, INR, PCR-RFLP

1. Introduction

Both warfarin and acenocoumarol are used in various disorders such as deep venous thrombosis, pulmonary embolism, atrial fibrillation and artificial heart valves (*1,2*). They have improved the prognosis of patients with thromboembolic disease. An individual's response to coumarin derivatives depends on several factors. The non-genetic factors include age, gender, body mass index (BMI), diet and interacting drugs (*1*).

Among the genetic factors, the cytochrome P450 (CYP) system and vitamin K epoxide reductase complex subunit 1 (VKORC1) play a key role in the drug metabolism (*3*). The cytochrome P450s are a multigene family of enzymes found predominantly in the liver and are responsible for the metabolic elimination of most of the drugs. CYP2C9 is the second family of cytochrome P450 system. To date, 65 *CYP2C9* variant alleles have been reported (*4*). *CYP2C9*1* is the wild-type allele. There are two

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important single nucleotide polymorphisms (SNPs), the *CYP2C9*2* (C430T, exon 3) associated with a functionally important Arg144Cys substitution and the *CYP2C9*3* (A1075C, exon 7) associated with Ile359Leu substitution. Both variants are associated with a reduced enzymatic activity and hence a lower drug requirement (*5*). Acenocoumarol and warfarin are metabolized by CYP2C9 enzyme system. It has been shown that *CYP2C9* gene polymorphism contributes up to 15% variability in case of warfarin and about 5% in case of acenocoumarol (*2*).

VKORC1 is the target enzyme of oral anticoagulants. The inhibition of this enzyme reduces the regeneration of active form of vitamin K from vitamin K epoxide reductase (*6*). Many polymorphisms have been found both in the coding and the non-coding regions of the *VKORC1* gene. *VKORC1* −1639G>A is a polymorphism in the promoter region, *VKORC1* 9041G>A is a polymorphism in the 3'UTR region and *VKORC1* 6009 C>T variant is polymorphism in the intron- 1 region of *VKORC1* gene. The presence of these polymorphisms are known to contribute up to 30% in the dose requirements of warfarin and acenocoumarol (*3,6*).

There are a few studies from India about these polymorphisms and their effects on patients on long term oral anticoagulation (*7-11*). In this study, the allele and genotypic frequencies of two of the *CYP2C9* gene polymorphisms; 430C>T and 1075A>C and three of the *VKORC1* gene polymorphisms; 1639G>A, 9041G>A and 6009C>T in 106 alleles of north Indian patients with valve replacement on acenocoumarol were determined and their effect on acenocoumarol dosing was studied. To the best of our knowledge, this is the first report of *VKORC1* 9041G>A and 6009C>T gene polymorphisms and their effect on acenocoumarol dosing from north India.

2. Materials and Methods

2.1. *Study subjects*

This was a prospective hospital based study on fifty-three patients who attended the out-patient clinic of the Department of Cardiology over a period of 1 year from September 2013 to August 2014 and gave consent for the study and fulfilled the inclusion criteria. Institutional ethics committee approval was obtained prior to the study. Written informed consent was obtained from the patients participating in this study. The inclusion criteria comprised patients with prosthetic heart valves, between 18-65 years of age on anticoagulation treatment with acenocoumarol for prevention of thromboembolism. The mean daily maintenance dose (mg/day) of acenocoumarol was defined as "the dose of acenocoumarol for minimum of 3 months with two or more consecutive INR measurements done at least 7 days apart being within target range (2 to 3.5) to prevent thromboembolism" (*12*). Data on participants' age, height, weight, body mass index, medication history, INR values, and acenocoumarol dose was recorded. The exclusion criteria comprised concomitant therapy with drugs potentially interacting with acenocoumarol, liver or renal dysfunction, pregnant and lactating women, smokers, chronic alcoholics and patients on warfarin.

2.2. *INR testing*

INR testing was performed on peripheral blood on the fully automated STA-R Evolution coagulation analyzer on citrated blood a per the manufacturer's instruction. The reagent used to determine the PT had an ISI value of 0.9-1.1 (Diagnostica Stago (STA) Neoplastin R, Asnieres, France). The expected INR range was 2-3.5.

2.3. *PCR-RFLP*

DNA was isolated from two ml of EDTA venous blood using Midi-Kit method (QIAGEN amp DNA Midi Kit, California, USA) as per instruction provided by the manufacturer. PCR-RFLP was performed using primers and PCR conditions as described previously with modifications (given in supplementary material) (*13,14*).

2.4. *Statistical analysis*

Normality of quantitative data was checked by measures of Kolmogorov Smirnov tests of normality. The patient's data was normally distributed hence, discrete categorical data is presented as n (%) and continuous data is presented as mean ± standard deviation (SD) and the confidence intervals (CI) were calculated, as appropriate. The allele and genotype frequencies for *CYP2C9* and *VKORC1* gene polymorphisms were expressed as percentage (%) and CI. Categorical data for *e.g*. age and dose was compared by Chi-square or Fisher's exact test. All statistical tests were two-sided and performed at a significance level of $p = 0.05$. All analyses were performed using SPSS for Windows (version 17.0; SPSS Inc., Chicago, IL, USA). To find independent predictors of dose per week of acenocoumarol both multivariate regression analysis and bivariate logistic regression analysis were applied. Pearson's coefficient of regression was applied to analyze the relation of dose with different variables.

3. Results

A total of 53 patients from north India were enrolled in the study. All patients had undergone heart valve replacement surgery and were receiving regular oral anticoagulant therapy in the form of acenocoumarol. The primary indication for valve replacement was

rheumatic heart disease in 92% cases (49/53), the other indications were bicuspid aortic valve in 2 cases and degenerated aortic valve and dilated cardiomyopathy in 1 case each. Single heart valve replacement was seen in 81% cases (43/53) and double valve in 19% cases (10/53). The mean INR was 2.42 (range, 2-3) and the mean follow-up period was 3.35 years (range, 1-12 years). The mean dose of acenocoumarol was 17.5 mg/week. For assessing the association of drug dose in relation to the gene polymorphisms, the patients were classified based on mean dose of acenocoumarol into 2 arbitrary groups; low dose (≤ 17.5 mg/week) and high dose (> 17.5 mg/week) groups. The characteristics of patients with respect to dosage of acenocoumarol are given in Table1.

The allele and genotype frequencies of both *CYP2C9* and *VKORC1* gene polymorphisms were determined (Table 2). The allele frequencies were in Hardy-Weinberg equilibrium.

The mean weekly dose of acenocoumarol in mg/week was lower in a large proportion of the patients with the mutant allele for *CYP2C9*2* (C430T), *CYP2C9*3* (A1075C) and *VKORC1*2* (G1639A) gene polymorphisms when compared with those with wild type allele and the mean dose of acenocoumarol in mg/week was higher in patients with the mutant allele for *VKORC1*3* (G9041A) and *VKORC1*4* (C6009T) gene polymorphisms when compared with those with wild

Table 1. Characteristics of patients (*n* = 53) with respect to dosage of acenocoumarol

Parameters	Variables	Low dose of acenocoumarol (≤ 17.5 mg/week), *n* = 33	High dose of acenocoumarol (> 17.5mg/week), *n* = 20	*p* value
Age in years, mean ± SD	38.1 ± 13.3	38.3 ± 14	37.7 ± 12.2	0.88
Sex				
Males *n* (%)	29 (55)	18	9	0.9
Females *n* (%)	24 (45)	15	11	
Height in cm, mean ± SD	162.3 ± 9	161.9 ± 7.1	162.8 ± 11.7	0.7
Weight in kg, mean ± SD	60.2 ± 11.5	58.9 ± 11.0	62.30 ± 12.2	0.3
Body mass index in kg/m^2, mean ± SD	22.9 ± 4.2	22.5 ± 4.1	23.5 ± 4.3	0.6
Patients taking concomitant amiodarone, *n* (%)	8 (15.2)	7	1	*
Patients taking concomitant atorvastatin, *n* (%)	2 (3.8)	2	0	*

*small number of patients

Table 2. Allele and genotype frequencies of *CYP2C9* and *VKORC1* gene polymorphisms

CYP2C9 Gene Polymorphism	Alleles	Number of alleles (*n* = 106)	Allele frequency in %	95% Confidence Interval in %
*2 (C430T)	C	100	94.33	88-97
	T	06	05.67	2.6-12
*3 (A1075C)	A	88	83.01	75-89
	C	18	16.98	11-25
CYP2C9 Gene Polymorphism	Genotype	Number of subjects (*n* = 53)	Genotype frequency in %	95% Confidence Interval in %
*2 (C430T)#	CC	47	88.68	77-95
	CT	06	11.32	5.3-26
*3 (A1075C)	AA	36	67.92	55-79
	AC	16	30.18	19-44
	CC	01	01.88	3.3-9.9
VKORC1 Gene Polymorphism	Alleles	Number of alleles (*n* = 106)	Allele frequency in %	95% Confidence Interval in %
*2 (G1639A)	G	90	84.9	77-90
	A	16	15.1	9.5-23
*3 (G9041A)	G	30	28.3	21-38
	A	76	71.7	62-79
*4 (C6009T)	C	94	88.7	81-93
	T	12	11.3	6.6-19
VKORC1 Gene Polymorphism	Genotype	Number of subjects (*n* = 53)	Genotype frequency in %	95% Confidence Interval in %
*2 (G1639A)#	GG	37	69.81	56-80
	GA	16	30.19	19-44
*3 (G9041A)	GG	06	11.32	5.3-23
	GA	18	33.96	23-48
	AA	29	54.72	42-67
*4(C6009T)#	CC	41	77.36	64-86
	CT	12	22.64	13-36

#No mutant genotypes were found

Table 3. Association of *CYP2C9* and *VKORC1* gene polymorphisms with the likelihood of requiring a low (≤ 17.5mg/week) or a high dose (> 17.5mg/week) of acenocoumarol

CYP2C9 Gene Polymorphism	Genotype	Dose category in mg/week		*p* value
		Low dose, *n* = 33 (%)	High dose, *n* = 20 (%)	
*2 (C430T)	CC	28 (85)	19 (95)	0.28
	CT	05 (15)	01 (05)	
*3 (A1075C)	AA	20 (61)	16 (80)	0.19
	AC	12 (36)	04 (20)	
	CC	01 (03)	0	
VKORC1 Gene Polymorphism				
*2 (G1639A)	GG	20 (61)	17 (85)	0.07
	GA	13 (39)	03 (15)	
*3 (G9041A)	GG	06 (18)	0	0.09
	GA	09 (27)	09 (45)	
	AA	18 (55)	11 (55)	
*4 (C6009T)	CC	26 (79)	15 (75)	0.74
	CT	07 (21)	05 (25)	

type allele. However, the difference was not statistically significant (Table 3).

A haplotype analysis for each patient based on the individual's *CYP2C9* and *VKORC1* genotype was performed (Table 4). In the 106 alleles analysed for different *CYP2C9* polymorphisms, the most frequent haplotype observed was *1/*1 (56.6%) followed by *1/*3 (30.19%), *1/*2 (11.32%) and*3/*3 (1.88%). Homozygous mutant haplotype*3/*3 was found in one subject. The most frequent *VKORC1* gene haplotype was *3/*3 (54.7%) followed by *2/*3 (18.8%), *3/*4 (13.2%) and*2/*4 (9.4%). Haplotypes*1/*2 and *1/*3 were found in one subject each.

The most common combined haplotype for *CYP2C9* and *VKORC1* genes respectively was *1/*3 and*3/*3 respectively, seen in 23% (12/53) patients and the least common were haplotypes *1/*1 and *1/*3, *1/*2 and *1/*2, and *3/*3 and *3/*3 for *CYP2C9* and *VKORC1* genes respectively present in 1 patient each (Table 5).

Binary logistic regression analysis model revealed that both *CYP2C9*3*, and *VKORC1*2* (1639 G>A) gene polymorphisms contributed to the variability in low dose (≤ 17.5mg/week) of acenocoumarol, p value 0.038 and 0.025 respectively. Stepwise regression analysis model showed that *VKORC1*2* (1639 G>A) contributed to 7.6% to the mean dose variation of acenocoumarol (r^2 = 0.076, p = 0.025). Among the non-genetic factors, it was found that the dose requirements fell with increasing age however, the difference was not statistically significant. Significant dose differences were not seen with respect to the other non-genetic factors including age, gender and BMI.

Table 4. Distribution of various haplotypes of *CYP2C9* and *VKORC1* gene polymorphisms

Gene Polymorphism	Haplotype	*n* (%)
CYP2C9[#]	*1/*1	30 (56.6)
(C430T, A1075C)	*1/*2	6 (11.3)
	*1/*3	16 (30.2)
	*3/*3	1 (1.9)
VKORC1[##]	*1/*2	1 (1.9)
(G1639A,C6009T,G9041A)	*1/*3	1 (1.9)
	*2/*3	10 (18.9)
	*2/*4	5 (9.4)
	*3/*3	29 (54.7)
	*3/*4	7 (13.2)

[#]Haplotypes CYP2C9 *2/*2 and *2/*3 were not found in the study population. [##]Haplotypes VKORC1*1/*1, *1/*4, *2/*2 and *4/*4 were not found in the study population.

Table 5. Distribution of haplotype combinations of *CYP2C9* and *VKORC1* gene polymorphisms

CYP2C9	*VKORC1*	*n* = 53 (%)
*1/*1	*1/*3	01 (1.9)
*1/*1	*2/*3	08 (15)
*1/*1	*2/*4	03 (5.6)
*1/*1	*3/*3	11 (21)
*1/*1	*3/*4	07 (13)
*1/*2	*1/*2	01 (1.9)
*1/*2	*3/*3	05 (9.4)
*1/*3	*2/*3	02 (3.8)
*1/*3	*2/*4	02 (3.8)
*1/*3	*3/*3	12 (23)
*3/*3	*3/*3	1 (1.9)

p value 0.11 (ANOVA). Haplotypes *1/*1 and*1/*1, *1/*1 and *1/*2, *1/*1 and *1/*4, *1/*1 and *2/*2, *1/*1 and *4/*4, *1/*2 and *1/*1, *1/*2 and*1/*3 and *1/*2 and *1/*4 respectively for *CYP2C9* and *VKORC1* genes were not found.

4. Discussion

Warfarin and acenocoumarol are highly effective for the prevention and treatment of various thromboembolic disorders (*1*). Although warfarin is most used coumarin, acenocoumarol is also commonly used in many countries (*15*). It is well known that an individual's response to oral anticoagulants depends on several factors. These include genetic factors, non-genetic factors, ethnic factors and yet unknown factors (*1,2*).

The known genetic factors contributing to the variability in acenocoumarol dosing requirements include mainly *CYP2C9* and *VKORC1* gene polymorphisms with minor contributions from APOE and *CYP4F2* genes (*15*). In the current study two genetic polymorphisms of *CYP2C9* gene *i.e. CYP2C9*2* (C430T) and *CYP2C9*3* (A1075C) and three genetic polymorphisms of *VKORC1* gene *i.e. VKORC1*2* (G1639A), *VKORC1*3* (G9041A) and *VKORC1*4* (C6009T) were analysed. These polymorphisms are known to have a significant effect on the acenocoumarol dose requirements. To the best of our knowledge, this is the first study that has evaluated 5 single nucleotide polymorphisms in two genes, simultaneously evaluating their allele and genotypic frequencies and the role of two of these gene polymorphisms in influencing acenocoumarol dose requirements in north Indian patients.

The non-genetic factors contributing to the variability in acenocoumarol dosing requirements include age, gender, BMI, vitamin K intake, concurrent medications and patient compliance (*15*). Age has a varied impact on the dosage of acenocoumarol. Some studies have shown that the activity of the cytochrome (P450) enzyme system decreases with age and that dose requirements fell with advancing age, decreasing by 0.5 to 0.7 mg per decade between the ages of 20 to 90 years irrespective of genotype and patient's height (*16*) while, others have demonstrated the opposite trend (*17*). In the current study though the dosage decreased with age, the difference was not statistically significant (p = 0.49). It was also observed that gender did not associate significantly with acenocoumarol dose requirements in (p = 0.97). Though, some studies have shown that the daily maintenance dose of acenocoumarol for females was significantly higher than the males (*16*), others did not find any differences (*18*). Drug-drug interactions have also been associated with variations in the acenocoumarol dose requirements to obtain stable anticoagulation (*15*). The concurrent medications which were essential as a part of the treatment required by the patients were not excluded and their effect on acenocoumarol dosage was studied. In our study the bivariate analysis model did not show any significant differences in acenocoumarol dose requirement in patients receiving concomitant amiodarone (p = 0.12) or atorvastatin (p = 0.5), however considerable caution is to be taken in the interpretation of this observation since the numbers of patients on these drugs were very small. Some reports suggest that the use of statins together with acenocoumarol has led to a slight decrease in the average daily dose of the latter (*16*), whereas others did not show any significant differences between patients receiving concomitant medications and those without the medication (*19*). Excessive consumption of vitamin K-rich diets (*e.g.* green vegetables) reduces the anticoagulation effect of coumarin derivatives (*20*) and in contrast, the administration of certain antibiotics that interfere with the production of vitamin K by gut flora have been suggested to exaggerate the anticoagulation response to coumarins (*21*). In the current analyses, the dietary consumption of vitamin K was not considered, it was assumed that all patients had relatively stable vitamin K consumption, given that they had a stable INR. The BMI has been included as a parameter in various algorithms that predict acenocoumarol dose requirements (*15,19*). In the current analysis BMI was correlated with the acenocoumarol dose requirements by categorizing the patients into high and low dose groups. No significant correlation was found between BMI of the patients and acenocoumarol dose requirements (p = 0.6). Compliance is yet another factor that effects acenocoumarol dose requirement. Drug dosage required to achieve an anticoagulation response may vary in a non-compliant patient and hence, the time required to achieve stable INR is more (*9*). All patients enrolled in our study had an apparently good compliance as they had a stable INR. This may be the result of good patient counseling well before starting anticoagulation therapy by the clinicians. The effect of non-genetic factors including age, gender, BMI and concurrent medications did not significantly relate to the drug dosage in this study group.

The genetic factors such as *CYP2C9* and *VKORC1* gene polymorphisms account for 5% and 20% variability respectively in the dosing of acenocoumarol (*3,6*). The prevalence of these polymorphisms varies across different ethnic groups. In the current study, the allele frequencies of *CYP2C9*1,*2,*3* were 0.773, 0.056, 0.169, respectively. The allelic frequencies were in Hardy-Weinberg equilibrium. The allele frequency of *CYP2C9*2* gene polymorphism in north Indian patients was higher than in other Asian countries (0.029), African-Americans (0.028) and south Indians (0.025) but was lower than the Caucasians (0.151) (*22,23*). The allele frequency of *CYP2C9*3* (A1075C) gene polymorphism in north Indian patients was higher than the other Asian countries (0.039), African-Americans (0.020), south Indians (0.083) and Caucasian population (0.057) (*22,23*). The allele frequency was comparable to that of Romanians (0.155) and also Indians residing in Singapore (0.18) (*14,24*). Further, in north Indian patients the allele frequency of *CYP2C9*3* gene polymorphism (0.169) was more than the allele frequency of *CYP2C9*2* gene polymorphism (0.056). A study from India showed similar findings, while, other two from the same region showed the reverse trend (*7,8,10*).

It was found that the carriers of *CYP2C9*3* alleles had the lowest dose requirement followed by carriers of *CYP2C9*2* alleles. This observation is in concordance with other similar studies on the effects of *CYP2C9*2* and *CYP2C9*3* polymorphisms on acenocoumarol (*8,17*). Of the total 53 subjects enrolled in our study, 62% (33/53) constituted the low dose group while 38% (*20*) patients required a higher dose of acenocoumarol

to maintain a stable INR. The patients with *CYP2C9*2* or *CYP2C9*3* alleles required a lower dose of acenocoumarol than patients without this variant. A similar trend was observed on 113 Spanish patients on acenocoumarol (*25*). Thus, it appears that the presence of the variant allele *CYP2C9*2* or *CYP2C9*3* in our patients necessitates a lower dose of acenocoumarol. An anticipated side effect of fixed dosage administration protocols is an increased risk of bleeding. None were documented in the course of this study.

Three different polymorphisms of *VKORC1* gene were studied, of which two, *VKORC1*3* (G9041A) and *VKORC1*4* (C6009T) have not previously been reported from north India. The allele frequency of *VKORC1*2* gene polymorphism was 0.15, similar to one reported in Malaysian Indians (0.14) (*26*). This was higher than the allele frequencies obtained in African-Americans (0.108) but lower than the Chinese (0.95), Caucasians (0.40) and the Israelis (0.41) (*14,23,27*). Among other studies from northern India the frequency of this polymorphism varied from 0.13-0.17 which was comparable to our study (8,10,11). The frequency of this polymorphism was lower at 0.079 in a study from South India while another study involving South Indian patients had results similar to this study at 0.14 (*10,22*). The presence of this gene polymorphism is associated with a lower dose of anticoagulants (*28*). *VKORC1*3* gene polymorphism was the most frequent SNP prevalent in our study with an allele frequency of 0.72. This polymorphism is also prevalent in the Tamil population (0.83) of southern India and is the most frequent one in the African population (0.43) and is also common among Caucasians (0.38) and Israelis (0.37) however, it is less prevalent in Chinese population (0.04) (*9,25,27,29*). Patients with this polymorphism are fast acetylators hence, would require a higher dose (*9*). The allele frequency of *VKORC1*4* gene polymorphism was 0.11. This SNP is less common in Chinese (0.01), 0.20 in Caucasians and 0.18 in Israelis (*14,27,28*). The patients with this polymorphism are also fast acetylators hence, would require a higher dose. Currently there is paucity of data available on the latter two and it would be of interest to determine similarities or differences in the diverse Indian population. We were unable to demonstrate significant differences studied, though it is known that the categorization into a low-dose and a high-dose haplotype group is clinically helpful to prevent the risk of under or over anticoagulation (*6,28*).

The effect of the *VKORC1* gene polymorphisms on drug dosage was studied. The presence of *VKORC1*2* gene polymorphism, was associated with lower doses of acenocoumarol whilst patients with *VKORC1*3* or *VKORC1*4* alleles required a higher dose of acenocoumarol than patients without this variant. In this study stepwise regression analysis model showed that *VKORC1*2* contributed to 7.6% to the variation of acenocoumarol dosage (r^2 = 0.076, p = 0.025). A study involving Caucasians has shown that *VKORC1*2* explained 17.6% of the dose variations of acenocoumarol (*16*). The first acenocoumarol dosing algorithm involved *VKORC1* and *CYP2C9* gene variants and clinical factors such as age, BMI and interacting drugs and also included *CYP4F2* and *APOE* gene variants and explained 60.6% of the total variability in the acenocoumarol dose needed to obtain a stable INR (*15*).

The limitations of this study were small sample size, exclusion of real life variables like pregnancy, smoking, alcohol intake, the inclusion of a restricted INR range and absence of data pertaining to other genetic polymorphisms that affect the metabolism of acenocoumarol and therefore, its dosage. Nevertheless, this preliminary study adds to the prevalence data of the *VKORC1* gene polymorphisms G9041A and C6009T that have hitherto not been reported from north India.

In the current study involving *CYP2C9* and *VKORC1* gene polymorphisms in north Indians on acenocoumarol with mechanical prosthetic valves, the *2 (C430T) polymorphism of *CYP2C9* and *3 (G9041) polymorphism of *VKORC1* was the most common. The high prevalence of *VKORC1* 9041A gene polymorphism in this sample population is a novel finding. As has been previously described, low dosage were associated with wild types of *VKORC1*2* and mutant types of *CYP2C9*2* and *CYP2C9*3*. Significant dose differences were not seen among the haplotypes and with respect to non-genetic factors. Further studies on larger populations are required to confirm the findings obtained in this North Indian cohort.

References

1. James AH, Britt RP, Raskino CL, Thompson SG. Factors affecting the maintenance dose of warfarin. J Clin Pathol. 1992; 45:704-706.
2. Leung AY, Chow HC, Kwong YL, Fung AT, Chow WH, Yip AS, Liang R. Genetic polymorphism in exon 4 of cytochrome P450 *CYP2C9* may be associated with warfarin sensitivity in Chinese patients. Blood 2001; 98:2584-2587.
3. Bodin L, Verstuyft C, Tregouet DA, Robert A, Dubert L, Funck-Brentano C, Jaillon P, Beaune P, Laurent-Puig P, Becquemont L, Loriot MA. Cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase (*VKORC1*) genotypes as determinants of acenocoumarol sensitivity. Blood. 2005; 106:135-140.
4. Wolf CR, Smith G. Pharmacogenetics. Br Med Bull. 1999; 55:366-386.
5. Thijssen HH, Flinois JP, Beaune PH. Cytochrome P4502C9 is the principal catalyst of racemic acenocoumarol hydroxylation reactions in human liver microsomes. Drug Metab Dispos. 2000; 28:1284-1290.
6. Rieder MJ, Reiner AP, Gage BF, Nickerson DA, Eby CS, McLeod HL, Blough DK, Thummel KE, Veenstra DL, Rettie AE. Effect of *VKORC1* haplotypes on transcriptional regulation and warfarin dose. N Engl J Med. 2005; 352:2285-2293.

7. Kaur A, Khan F, Agrawal SS, Kapoor A, Agarwal SK, Phadke SR. Cytochrome P450 (*CYP2C9*2,*3*) & vitamin-K epoxide reductase complex (*VKORC1-1639G<A*) gene polymorphisms & their effect on acenocoumarol dose in patients with mechanical heart valve replacement. Indian J Med Res. 2013; 137:203-209.
8. Rathore S, Agarwal S, Pande S, Mittal T, Mittal B. Frequencies of *VKORC1* -1639 G>A, *CYP2C9*2* and *CYP2C9*3* genetic variants in the Northern Indian population. BioSci Trends. 2010; 4:333-337.
9. Madhan S, Kumar D, Kumar D, Balachander J, Adithan C. Effect of *CYP2C9* and *VKORC1* genetic polymorphisms on warfarin dose requirement in south Indian population. Indian J Physiol Pharmacol. 2013; 57:308-317.
10. Nahar R, Deb R, Saxena R, Puri R, Verma I. Variability in *CYP2C9* allele frequency: A pilot study of its predicted impact on warfarin response among healthy South and North Indians. Pharmacol Rep. 2013; 65:187-194.
11. Shalia K, Doshi S, Parikh S, Pawar P, Divekar S, Varma SP, Mehta R, Doctor T, Shah VK, Saranath D. Prevalence of *VKORC1* and *CYP2C9* gene polymorphisms in Indian population and its effect on Warfarin response. JAPI. 2012; 60:34-38.
12. Hirsh J, Dalen JE, Anderson DR, Poller L, Bussey H, Ansell J, Deykin D. Oral anticoagulants: Mechanism of action, clinical effectiveness, and optimal therapeutic range. Chest. 2001; 119(suppl):8S-21S.
13. Sullivan-Klose TH, Ghanayem BI, Bell DA, Zhang ZY, Kaminsky LS, Shenfield GM, Miners JO, Birkett DJ, Goldstein JA. The role of the *CYP2C9*-Leu359 allelic variant in the tolbutamide polymorphism. Pharmacogenetics. 1996; 6:341-349.
14. Sipeky C, Csongei V, Jaromi L, Safrany E, Polgar N, Lakner L, Szabo M, Takacs I, Melegh B. Vitamin K epoxide reductase complex 1 (*VKORC1*) haplotypes in healthy Hungarian and Roma population samples. Pharmacogenomics. 2009; 10:1025-1032.
15. Borobia A, Lubomirov R, Ramirez E, Lorenzo A, Campos A, Munroz-Romo R, Fernández-Capitán C, Frías J, Carcas AJ. Acenocoumarol dosing algorithm using clinical and pharmacogenetic data in Spanish patients with thromboembolic disease. PLoS One. 2012; 7:1-10.
16. Pop T, Vesa S, Trifa A, Crişan S, Buzoianu A. An acenocoumarol dose algorithm based on a South-Eastern European population. Eur J Clin Pharmacol. 2013; 1:1551-1553.
17. Sconce E, Khan T, Wynne H, Avery P, Monkhouse L, King BP, Wood P, Kesteven P, Daly AK, Kamali F. The impact of *CYP2C9* and *VKORC1* genetic polymorphism and patient characteristics upon warfarin dose requirements: Proposal for a new dosing regimen. Blood. 2005; 106:2329-2333.
18. Arboix M, Laporte J, Frati M, Rutllan M. Effect of age and sex on acenocoumarol requirements. Br J Clin Pharmac. 1984; 18:475-479.
19. Van Schie RM, Wadelius MI, Kamali F, Daly AK, Manolopoulos VG, de Boer A, Barallon R, Verhoef TI, Kirchheiner J, Haschke-Becher E, Briz M, Rosendaal FR, Redekop WK, Pirmohamed M, Maitland van der Zee AH. Genotype-guided dosing of coumarin derivatives: The European pharmacogenetics of anticoagulant therapy (EU-PACT) trial design. Pharmacogenomics. 2009; 10:1687-1695.
20. Schurgers LJ, Shearer MJ, Hamulyak K, Stocklin E, Vermeer C. Effect of vitamin K intake on the stability of oral anticoagulant treatment: Dose-response relationships in healthy subjects. Blood. 2004; 104:2682-2689.
21. Takahashi H, Wilkinson G, Padrini R, Echizen H. *CYP2C9* and oral anticoagulation therapy with acenocoumarol and warfarin: Similarities yet differences. Clin Pharmacol Ther. 2004; 75:376-380.
22. Krishna Kumar D, Madhan S, Balachander J, Sai Chandran BV, Thamijarassy B, Adithan C. Effect of *CYP2C9* and *VKORC1* genetic polymorphisms on mean daily maintenance dose of acenocoumarol in South Indian patients. Thromb Res. 2013; 131:363-367.
23. Scott S, Khasawneh R, Kornreich I, Desnick R. Combined *CYP2C9*, *VKORC1* and *CYP4F2* frequencies among racial and ethnic groups. Pharmacogenomics. 2010; 11:781-791.
24. Lee SC, Ng SS, Oldenburg J, Chong PY, Rost S, Guo JY, Yap HL, Rankin SC, Khor HB, Yeo TC, Ng KS, Soong R, Goh BC. Interethnic variability of warfarin maintenance requirement is explained by *VKORC1* genotype in an Asian population. Clin Pharmacol Ther. 2006; 79:197-205.
25. Hermida J, Zarza J, Alberca I, Montes R, López M, Molina E. Differential effects of 2C9*3 and 2C9*2 variants of cytochrome P-450 *CYP2C9* on sensitivity to acenocoumarol. Blood. 2002; 99:4237-4239.
26. Gan G, Lee M, Subramaniam R, Lu L, Tai M, Phipps M. Allele and Genotype frequencies of *VKORC1* -1639G>A polymorphism in three different ethnic groups in Malaysia. As Pac J Mol Biol Biotechnol. 2012; 20:19-23.
27. Loebstein R, Dvoskin I, Halkin H, Vecsler M, Lubetsky A, Rechavi G, Amariglio N, Cohen Y, Ken-Dror G, Almog S, Gak E. A coding *VKORC1* Asp36Tyr polymorphism predisposes to warfarin resistance. Blood. 2007; 109:2477-2480.
28. Geisen C, Watzka M, Sittinger K, Steffens M, Daugela L, Seifried E, Müller CR, Wienker TF, Oldenburg J. *VKORC1* haplotypes and their impact on the inter-individual and interethnical variability of oral anticoagulation. Thromb Haemost. 2005; 94:773-779.

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Regression analysis of the risk factors for postoperative nosocomial infection in patients with abdominal tumors: experience from a large cancer centre in China

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Summary **Nosocomial infection is a common complication after abdominal oncology surgery. Aimed at finding its independent risk factors for prevention, all the patients who underwent abdominal oncology surgery were summarized from March 1st, 2010 to March 1st, 2013 from the oncology surgery department, Beijing Shijitan Hospital. The investigated variances were patients' information including admission number, sex, age, diabetes, diagnosis, length of stay, American society of anesthesiologists (ASA) grade, surgery time, number of drainage tubes. Comparisons were taken between the infected cases and non-infected cases for retrospective logistic regression analysis. 4 variances including diabetes, preoperative hospitalization time ≥ 6 days, surgery time ≥ 230 minutes, ASA grade ≥ III were found out to be related to nosocomial infection after surgery. The 4 variances mentioned above were risk factors for nosocomial infection after surgery.**

Keywords: Nosocomial infection, regression analysis, oncology surgery

1. Introduction

In patients who underwent surgical procedures, the natural barrier against bacteria was broken by surgical trauma or invasive procedures such as tracheal intubation under general anesthesia, central venous cannulation, urethral catheterization and gastrointestinal decompression (*1-4*). Long-term postoperative application of broad-spectrum antibiotics also increased the possibility of dysbacteriosis (*5,6*). Some scholars investigated the mortality in patients experiencing postoperative nosocomial infection in the departments of general surgery. The results showed 7.5% mortality in patients with single-pathogen nosocomial infection and a mortality of 17.1% in patients with multiple-pathogen mixed infection, which is higher than the average postoperative mortality of 0.53% (*7*).

The patients who underwent surgical operations were a high-risk population for nosocomial infection. Based on analysis of post-surgery patients in 30 countries by International Nosocomial Infection Control Consortium, the nosocomial infection rate was 2.88% (*8*). Some analyses of risk factors have been reported to reduce and control the occurrence of such complications previously (*9,10*). The primary objective of our study was to identify the independent risk factors of postoperative nosocomial infection. Subsequently we can manage patients by different postoperative infection risk levels, and establish a set of effective protocols for the prevention and control of infections in high-risk populations.

2. Materials and Methods

2.1. *Patients*

All postoperative patients with abdominal digestive tumors underwent surgical procedures in Capital Medical Cancer Centre, Beijing Shijitan Hospital between March 1st, 2010 and March 1st, 2013 were included. The analytical contents included the gender, age, diabetes mellitus, diagnosis, preoperative hospitalization days, American society of anesthesiologists (ASA) grade, type

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Table 1. Percentiles of continuous variables in presumptive risk factors

Risk factor	5%	10%	25%	50%	75%	90%	95%
Age	38.85	43.00	51.00	58.00	68.00	74.00	78.00
Preoperative hospitalization days	2.00	2.00	3.00	4.00	6.00	10.00	12.00
Operation time	65.00	90.00	120.00	170.00	230.00	280.00	315.00
Number of drainage tubes	1.00	2.00	2.00	2.00	2.00	3.00	3.00
Postoperative hospitalization days	5.00	8.00	9.00	11.00	15.00	20.00	27.30

In order to take the logistic regression analysis, all continuous variables were divided into binomial variables using 75% as the cut-off point. Accordingly, age ≥ 68 years, preoperative hospitalization stay≥ 6 days, operation time ≥ 230 minutes, drainage tubes ≥ 3, ASA ≥ class III were the presumptive risk factors.

of surgery, number of drainage tubes, and central venous catheters to identify the independent risk factors. The presumptive risk factors referred to the article published by "National Nosocomial Infection Surveillance" of the U.S. Center for Disease Control in 2003 (*1*).

2.2. *Inclusion and grouping criteria*

The included patients were randomized into the observation group or the control group. The diagnosis of patients with nosocomial infection was based on the diagnostic criteria for nosocomial infection issued by the ministry of health in 2001 (*2*). The patients who suffered from nosocomial infection after surgery were included in the observation group. The patients who underwent surgical procedures during the same period without nosocomial infection were included in the control group.

2.3. *Collection and identification of microbe samples*

The collection of pathogenic microbe samples complied with "Good Laboratory Practice". The identification employed VITEK-2 automated microbial identification system made by French bioMérieux; the drug sensitivity test followed National Committee for Clinical Laboratory Standard.

2.4. *Determination of the statistical indexes*

With reference to the statistical index processing method reported by Daniel N Nan (*5*), the means of all variables were calculated and all variables were divided into binomial variables using 75% as the cut-off point. Accordingly, age ≥ 68 years, preoperative hospitalization stay ≥ 6 days, operation time ≥ 230 minutes, drainage tubes ≥ 3, ASA ≥ class III and diabetes mellitus were the presumptive risk factors (Table 1). Their presence and absence were regarded as binomial variables.

2.5. *Statistical analysis*

These cases were calculated by a retrospective logistic regression analysis method. SPSS (version 19.0.2; SAS Institute Inc., Cary, NC) software was employed for statistical analysis.

Table 2. Diagnostic frequency of the included patients

Diagnosis	Frequency	Percent	Valid Percent
Carcinoma of the distal bile duct	71	5.9	5.9
Carcinoma of the gallbladder	12	1.0	1.0
Hepatocellular carcinoma	69	5.8	5.8
Hilar cholangiocarcinoma	15	1.3	1.3
Periampullary carcinoma	8	0.7	0.7
Colon cancer	251	21.0	21.0
Malignant lymphoma of the colon	1	0.1	0.1
Duodenal cancer	33	2.8	2.8
Gastric cancer	401	33.5	33.5
Carcinoma of pancreatic body and tail	4	0.3	0.3
Carcinoma of pancreatic head	61	5.1	5.1
Sigmoid colon cancer	40	3.3	3.3
Colorectal cancer	230	19.2	19.2
Total	1196	100.0	100.0

Table 3. Diagnostic frequency of the infected patients

Diagnosis	Frequency	Percent	Valid Percent
Cholangiocarcinoma	7	5.9	5.9
Hepatocellular carcinoma	6	5.1	5.1
Hilar cholangiocarcinoma	2	1.7	1.7
Colon cancer	19	16.1	16.1
Duodenal cancer	7	5.9	5.9
Gastric cancer	25	21.2	21.2
Carcinoma of pancreatic head	3	2.5	2.5
Pancreatic cancer	9	7.6	7.6
Colorectal cancer	9	7.6	7.6
Total	118	100.0	100.0

3. Results

During the study period, 1,196 consecutive patients underwent surgeries for abdominal digestive system malignancies (Table 2). There were 87 patients who suffered from infections including 22 cases of mixed infections. The infection person-times were 118 (Table 3).

The overall postoperative infection rate was 7.3%. In all patients who suffered from postoperative nosocomial infection, the major infection sites were distributed as follows: 38.1% pulmonary infection, 12.7% biliary tract infections, and 11.9% surgical site infection (Table 4).

Nosocomial infection was mainly caused by Gram-negative bacteria which accounted for 45.8% of

Table 4. Sampling sites of nosocomial infection pathogens

Sample	Frequency	Percent	Valid Percent
Stool culture	1	0.8	0.8
Bile	15	12.7	12.7
Catheter	4	3.4	3.4
Feces	4	3.4	3.4
Ascites	7	5.9	5.9
Urine	3	2.5	2.5
Pus	1	0.8	0.8
Incision	1	0.8	0.8
Sputum	45	38.1	38.1
Pleural fluid	3	2.5	2.5
Blood culture	8	6.8	6.8
Pharyngeal swabs	12	10.2	10.2
Drainage fluid	14	11.9	11.9
Total	118	100.0	100.0

Table 5. Type of nosocomial infection pathogens

Pathogen	Frequency	Percent
Gram-positive	36	30.5
Gram-negative	54	45.8
Fungus	28	23.7
Total	118	100.0

nosocomial infection (Table 5). Among them *Escherichia coli* accounted for (22/54) 40.7% of these Gram-negative bacteria; and these pathogens were resistant to the fourth-generation cephalosporins and sensitive to imipenem. *Acinetobacter baumannii* accounted for 20.5% (11/54) of the Gram-negative bacteria; the drug resistant spectrum and sensitivity were identical to *Escherichia coli*; and some strains were highly sensitive to amikacin. The Gram-negative bacteria mainly came from peritoneal drainage fluid, bile, *etc*.

Gram-positive bacteria accounted for 30.5% of nosocomial infection. Among them methicillin-resistant staphylococcus aureus (MRSA) accounted for 58.3% (21/36) of the Gram-positive bacteria. They were all sensitive to vancomycin; and no vancomycin resistant staphylococcus aureus was identified. The bacteria were mainly from sputum and blood cultures. Fungi accounted for 23.7% and mainly came from pharyngeal swabs and sputum culture (Table 6).

The results of binomial logistic regression analysis of the variables are shown as follows (Table 7). The four variables of significance were diabetes mellitus, operation time ≥ 230 minutes, preoperative hospitalization time ≥ 6 days, and ASA classification ≥ class III. However, male gender, $p = 0.968$; drainage tubes ≥ 3, $p = 0.763$ and age ≥ 68 years, $p = 0.120$ didn't show statistical significance at $p = 0.05$ level.

Multivariate analysis of the relative risk of these four independent risk factors showed that all the odds ratios were greater than 1 within a 95% confidence interval. The results once again demonstrated the reliability of these 4 variables as the nosocomial infection risk factors (Table 8).

Table 6. Strains of nosocomial infection pathogens

Pathogen	Frequency	Percent
MRSA	21	17.8
Candida albicans	13	11.0
Acinetobacter baumannii	10	8.5
Acinetobacter	1	0.8
Enterococcus gallinarum	1	0.8
Rough-type bacteria	1	0.8
Escherichia coli	22	18.6
Klebsiella pneumoniae	7	5.9
Enterococcus faecalis	6	5.1
Citrobacter freundii	1	0.8
Gram-positive bacteria	1	0.8
Gram-positive coccus	2	1.7
Candida glabrata	4	3.4
Saccharomyces	1	0.8
Raoultella ornithinolytica	1	0.8
Enterobacter agglomerans	1	0.8
Monilia krusei	4	3.4
Candida	1	0.8
Enterococcus casselifavus	1	0.8
Staphylococcus haemolyticus	1	0.8
Stenotrophomonas maltophilia	5	4.2
Pseudomonas aeruginosa	3	2.5
Aeromonas veronii	1	0.8
Enterobacter cloacae	2	1.7
Enterococci cloacae	3	2.5
Fungal spores	4	3.4
Total	118	100.0

The average hospitalization time was 11 days after operation of abdominal malignancies. The 75 percentile days were 15 days (Table 1). Based on the linear regression results for infection and postoperative hospitalization time ≥ 15 days, the correlation between these two indexes had statistical significance. The analysis confirmed that postoperative nosocomial infection increased the hospitalization stay and the medical costs.

The 4 variables including diabetes mellitus, operation time ≥ 230 minutes, preoperative hospitalization stay ≥ 6 days, ASA classification ≥ class III are the risk factors for postoperative nosocomial infection in patients with tumors. Postoperative nosocomial infection increases the postoperative stay in the hospital.

4. Discussion

Our study and the other reports stressed the prevention and control of abdominal postoperative nosocomial infection. Surgical operation of abdominal tumors has a high fatality (*11*). In our study, 7.3% of the patients experienced postoperative nosocomial infection. Although the mortality was not calculated, the postoperative hospitalization stay ≥ 15 days was certainly correlated with nosocomial infection. Identification of the risk factors is a prerequisite for better preventive methods and to develop prospective plans for infection control and treatment.

Long-term metabolic disturbance in diabetes

Table 7. The results of binomial logistic regression analysis of each variable

Step	B	S.E.	Wald	Sig.	Exp.
Step 1					
SEX	-0.122	0.251	0.234	0.628	0.886
DIABETES	1.028	0.299	11.857	0.001	2.796
AGE	0.431	0.268	2.587	0.108	1.539
SURTIME	1.268	0.279	20.604	0.000	3.555
DRAIN	-0.116	0.322	0.129	0.719	0.890
ASA	0.727	0.246	8.754	0.003	2.068
POSTSTAY	0.645	0.260	6.165	0.013	1.907
PRESURG	1.328	0.251	27.916	0.000	3.773
Constant	-4.274	0.457	87.309	0.000	0.014
Step 2					
SEX	-0.123	0.251	0.239	0.625	0.884
DIABETES	1.042	0.296	12.375	0.000	2.835
AGE	0.430	0.268	2.571	0.109	1.537
SURTIME	1.231	0.261	22.272	0.000	3.425
ASA	0.728	0.246	8.788	0.003	2.071
POSTSTAY	0.626	0.255	6.038	0.014	1.871
PRESURG	1.330	0.251	27.987	0.000	3.783
Constant	-4.276	0.457	87.489	0.000	0.014
Step 3					
DIABETES	1.025	0.294	12.127	0.000	2.786
AGE	0.416	0.266	2.433	0.119	1.515
SURTIME	1.228	0.261	22.159	0.000	3.415
ASA	0.733	0.245	8.942	0.003	2.082
POSTSTAY	0.619	0.254	5.923	0.015	1.857
PRESURG	1.352	0.248	29.822	0.000	3.867
Constant	-4.451	0.292	232.094	0.000	0.012
Step 4					
DIABETES	1.081	0.291	13.770	0.000	2.946
SURTIME	1.130	0.252	20.175	0.000	3.097
ASA	0.789	0.242	10.619	0.001	2.202
POSTSTAY	0.699	0.249	7.884	0.005	2.012
PRESURG	1.319	0.246	28.867	0.000	3.740
Constant	-4.325	0.275	246.518	0.000	0.013

SEX, DIABETES, AGE, SURTIME, DRAIN, ASA, POSTSTAY, PRESURG stood for "male", "diabetes", "age ≥ 68 years old", "operation time ≥ 230 minutes", "drain ≥ 3", "ASA grade ≥ 3", "hospital stay post-surgery ≥ 15days", "hospital stay pre-surgery≥ 6 days". Binomial logistic regression analysis of multiple variables was taken. The result showed that the four variables including diabetes mellitus, operation time ≥ 230 minutes, hospital stay pre-surgery ≥ 6 days, and ASA classification ≥ class III had statistical significance.

Table 8. Odds Ratio of the nosocomial infection risk factors at 95% confidence interval

Iterm of Odds Ratio for infection	Mean Value	Lower Value	Higher Value
For cohort with operation time > 230 minutes = 1	3.481	2.236	3.481
For cohort with ASA > 2 = 1	1.901	1.225	1.901
For cohort with preoperative hospitalization time > 6 days = 1	8.407	5.017	8.407
For cohort with diabetes mellitus = 1	3.453	2.041	3.453

mellitus caused multi-system and multi-organ impairments and undermines the immune function. Diabetic patients became the susceptible and an absolutely high-risk population for nosocomial infection (*12-14*). Generally, the interactions among multiple factors including metabolic disturbance, vasculopathy, and neuropathy effects were considered to be the main mechanisms of diabetes-complicated nosocomial infection (*15*).

The longer operation time and wound exposure time were likely to result in a higher possibility of contamination and higher probability of postoperative infection (*16*). Non-standardized surgical procedures, contamination of the surgical incision, surgeon's rough maneuvers, severe intraoperative bleeding and local tissue injuries could turn the surgical wounds into media for growth and multiplication of bacteria (*17*). Contamination in the operating room is closely related to surgical site contamination and postoperative nosocomial infection. It was reported that medical staff cell phones were severely contaminated with a strain detection rate of 95.5% and a HBsAg detection rate of

13.6% (*18*). The average microbial content conformity rate in the indoor air of the operating room was 78.57% for the first operation each day, 95.74% for successive operations, and 93.75% for successive operations following ventilation, wet cleaning and disinfection after the first operation (*19*).

It can be concluded from the statistical analysis results in this study that the extended hospitalization days are correlated with postoperative nosocomial infection. It can also be inferentially interpreted as increasing abdominal operation-related mortality.

The pathogen's drug resistance shows an ascending trend annually. It was reported that: the fungal resistance rate to fluconazole and itraconazole were 19% and 28% respectively in 2006; and 18% and 19%, respectively in 2007 (*20*). Drug resistance of G+ cocci, especially coagulase-negative staphylococcus, to penicillin was up to 84%~89%; the 2-year drug resistance of G+ cocci to cefotaxime, cefepime, ciprofloxacin, erythromycin, azithromycin and other antibiotics was up to ≥ 92%. The 2-year drug resistance of G- bacilli to ampicillin was up to ≥ 90% (*21*). The results of this study have confirmed this viewpoint. Therefore, the prophylactic application of antibiotics should be strictly standardized by the guidelines in order to reduce the overall induction of resistant bacteria at the nation wide level.

The 4 risk factors obtained from our research by binomial logistic regression analysis method has indicative significance. The 3 risk factors including diabetes mellitus, preoperative hospitalization stay ≥ 6 days and ASA grade ≥ III can easily be identified preoperatively. This can provide the basis not only for the management of high-risk patients but also for the formulation of measures to reduce infection complications. For the risk factor of operation time > 230 minutes, it is necessary to carry out serious surgery discussion, formulate the operation plan, and estimate all possible situations preoperatively in order to shorten the operation time as much as possible under the premise of quality assurance.

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References

1. Dramowski A, Madide A, Bekker A. Neonatal nosocomial bloodstream infections at a referral hospital in a middle-income country: Burden, pathogens, antimicrobial resistance and mortality. Paediatr Int Child Health. 2015; 35:265-272.
2. Rosenthal VD, Richtmann R, Singh S, *et al*. Surgical site infections, international nosocomial infection control consortium (INICC) report, data summary of 30 countries, 2005-2010. Infect Control Hosp Epidemiol. 2013; 34:597-604.
3. Leblebicioglu H, Erben N, Rosenthal VD, *et al*. International nosocomial infection control consortium (INICC) national report on device-associated infection rates in 19 cities of Turkey, data summary for 2003-2012. Ann Clin Microbiol Antimicrob. 2014; 13:51.
4. Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK. National healthcare safety network team, participating national healthcare safety network facilities. Antimicrobial-resistant pathogens associated with healthcare-associated infections: Annual summary of data reported to the national healthcare safety network at the centers for disease control and prevention, 2006-2007. Infect Control Hosp Epidemiol. 2008; 29:996-1011.
5. Nan DN, Fernández-Ayala M, Fariñas-Alvarez C, Mons R, Ortega FJ, González-Macías J, Fariñas MC. Nosocomial infection after lung surgery: Incidence and risk factors. Chest. 2005; 128:2647-2652.
6. Leblebicioglu H, Erben N, Rosenthal VD, Sener A, Uzun C, Senol G, Ersoz G, Demirdal T, Duygu F, Willke A, Sirmatel F, Oztoprak N, Koksal I, Oncul O, Gurbuz Y. Surgical site infection rates in 16 cities in Turkey: Findings of the international nosocomial infection control consortium (INICC). Am J Infect Control. 2015; 43:48-52.
7. Tserenpuntsag B, Haley V, Van Antwerpen C, Doughty D, Gase KA, Hazamy PA, Tsivitis M. Surgical site infection risk factors identified for patients undergoing colon procedures, New York State 2009-2010. Infect Control Hosp Epidemiol. 2014; 35:1006-1012.
8. Vilar-Compte D, Mohar A, Sandoval S, de la Rosa M, Gordillo P, Volkow P. Surgical site infections at the national cancer institute in Mexico: A case-control study. Am J Infect Control. 2000; 28:14-20.
9. Silva TN, de Marchi D, Mendes ML, Barretti P, Ponce D. Approach to prophylactic measures for central venous catheter-related infections in hemodialysis: A critical review. Hemodial Int. 2014; 18:15-23.
10. Chen KY, Ko SC, Hsueh PR, Luh KT, Yang PC. Pulmonary fungal infection: Emphasis on microbiological spectra, patient outcome, and prognostic factors. Chest. 2001; 120:177-84.
11. Gaynes RP, Culver DH, Horan TC, Edwards JR, Richards C, Tolson JS. Surgical site infection SSI rate in the United States, 1992-1998: The national nosocomial infections surveillance system basic SSI risk index. Clin Infect Dis. 2001; 33:69-77.
12. Kropec A, Schulgen G, Just H, Geiger K, Schumacher M, Daschner F. Scoring system for nosocomial pneumonia in ICUs. Intensive Care Med. 1996; 22:1155-1161.
13. Diederen BM, Wardle CL, Krijnen P, Tuinebreijer WE, Breederveld RS. Epidemiology of clinically relevant bacterial pathogens in a nurn center in the Netherlands between 2005 and 2011. J Burn Care Res. 2015; 36:446-453.
14. Bayramoglu G, Kaya S, Besli Y, Cakır E, Can G, Akıneden O, Aydin F, Koksal I. Molecular epidemiology and the clinical significance of Acinetobacter baumannii complex isolated from cerebrospinal fluid in neurosurgical intensive care unit patients. Infection. 2012; 40:163-172.
15. Takano K, Fuji S, Uchida N, Ogawa H, Ohashi K, Eto T, Sakamaki H, Morishima Y7, Kato K, Suzuki R, Fukuda T. Pre-transplant diabetes mellitus is a risk factor for non-relapse mortality, especially infection-related mortality,

after allogeneic hematopoietic SCT. Bone Marrow Transplant. 2015; 50:553-558.
16. Rotstein C, Cummings KM, Nicolaou AL. Nosocomial infection rates at an oncology center. Infect Control Hosp Epidemiol. 1988; 9:13-19.
17. Lee YL, Thrupp LD, Friis RH, Fine M, Maleki P, Cesario TC. Nosocomial infection and antibiotic utilization in geriatric patients: A pilot prospective surveillance program in skilled nursing facilities. Gerontology. 1992; 38:223-232.
18. Smith EB, Raphael IJ, Maltenfort MG, J Arthroplasty. The effect of laminar air flow and door openings on operating room contamination. J Arthroplasty. 2013; 28:1482-1485.
19. Salmon S, Truong AT, Nguyen VH. Health care workers' hand contamination levels and antibacterial efficacy of different hand hygiene methods used in a Vietnamese hospital. Am J Infect Control. 2014; 42:178-181.
20. Richard VS, Mathai E, Cherian T. Role of anaesthetic equipment in transmitting nosocomial infection. J Assoc Physicians India. 2011; 49:454-458.
21. Mori-Yoshikawa N, Ohmagari N, Kirikae T. Organization of nosocomial infection control measures and local networks for infectious disease control in middle-scale hospitals in Japan. Jpn J Infect Dis. 2014; 67:379-381.

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Brief Report

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An aqueous extract from toad skin prevents gelatinase activities derived from fetal serum albumin and serum-free culture medium of human breast carcinoma MDA-MB-231 cells

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Summary **An aqueous extract from toad skin, cinobufacini, has been known to possess anticancer ability. The present study examined effect of toad skin extract on activity of gelatinases including matrix metalloproteinases-2 and -9 which play an important role in invasion of carcinoma cells. Gelatinase activities derived from fetal serum albumin and culture medium of human breast carcinoma cell line MDA-MB-231 were significantly prevented in the presence of toad skin extract. The inhibitory activity was found in water-soluble fraction of the extract prepared by the Bligh & Dyer method but not in $CHCl_3$-soluble lipid fraction. These results suggest that an aqueous extract from toad skin contains a water-soluble substance possessing a potent ability to prevent gelatinase activity. In conclusion, the water-soluble substance in toad skin extract cinobufacini may be able to regulate cancer cell migration accelerated by matrix metalloproteinases.**

Keywords: Toad skin extract cinobufacini, gelatinase, matrix metalloproteinase, cancer

1. Introduction

Carcinoma cells arise in epithelial tissues express extracellular matrix-degrading enzymes such as matrix metalloproteinases (MMPs) and invade into inner tissues while degrading basement membranes and stromal tissues (*1*). Of many types of MMPs, MMP-2 and -9, both of which possess gelatinase activity, are important to degrade basement membrane (*1,2*). Since the degradation of basement membrane is thought to be the initial step of metastasis, efforts to find and create compounds that can prevent gelatinase activity have proceeded (*3-6*).

An aqueous extract from the skin of toad *Bufo bufo* gargarizans Cantor, which is known as a source of the Chinese traditional medicine cinobufacini (*7*), has been focused in anticancer studies (*8,9*). Especially, a series of bufosteroids including bufalin, cinobufagin, and regibufogenin, which are ingredients of the toad skin extract and toad venom, has been found apoptosis-inducing ability against cancer cells *via* cell signaling pathways (*8,10-12*). Recently, the toad skin extract cinobufacini has been clinically applied to patients with cancer (*13-15*).

Our previous study indicated that the toad skin extract possessed not only cell toxicity such as apoptosis-inducing ability but also cancer cell migration-preventing ability *in vitro* (*16*). Considering that MMPs including gelatinases have an important role in cancer cell migration, it would be valuable to investigate whether the toad skin extract can inhibit gelatinase activity. Here, we describe that a type of water-soluble substance but not lipid such as bufosteroids in the toad skin extract could inhibit gelatinase activity.

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2. Materials and Methods

2.1. *Reagents*

Toad skin extract cinobufacini was kindly provided by Anhui Jinchan Biochemical Co., Ltd., Anhui, China. Bufosteroids such as bufalin and cinobufagin were obtained from Cosmo Bio, Tokyo, Japan and another bufosteroid regibufogenin was from Wako Pure Chemical Industries, Osaka, Japan. Fetal serum albumin (FCS) was obtained from GE Healthcare Life Sciences, HyClone Laboratories, Logan, UT, USA. Galardin (GM6001; *N*-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide) was obtained from Sigma-Aldrich Japan, Tokyo, Japan. All the chemicals used were of analytical grade.

2.2. *Cell culture*

Human breast carcinoma cell line MDA-MB-231 was obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) supplemented with penicillin-streptomycin, fungizone (Invitrogen), and 2 mM glutamine at 37°C in a 5% CO_2 atmosphere as described previously (*17*). Before use, the cells were cultured in medium containing 0.1% bovine serum albumin instead of FCS for 24 h at 37°C. The culture medium was centrifuged and the supernatant was subjected to experiments as a source of gelatinase.

2.3. *Gelatin zymography*

MMP species in FCS and culture supernatant of MDA-MB-231 cells were detected using gelatin zymography (*18*). Samples were mixed with the same volume of sample buffer (125 mM Tris-HCl, pH 6.8, containing 4% SDS, 10% glycerol, and 0.01% bromophenol blue). Fifteen µL each of the sample was applied to SDS-polyacrylamide gel electrophoresis (4% gel) in the presence of 10% gelatin (Nacalai Tesque, Kyoto, Japan) in the separating gel at 4°C. After the electrophoresis, the gel was treated with 1 mM 4-aminophenylmercuric acetate (Sigma-Aldrich Japan) for 20 h at 4°C to convert proenzymes such as proMMP-2 and proMMP-9 to active forms (*19,20*). The gel was washed with a renature buffer (50 mM Tris-HCl, pH 7.5, containing 2.5% Triton X-100, 0.15 M NaCl) for 15 min 3 times and then gently shaked in a developing buffer (50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, 0.7 mM $CaCl_2$, 1 mM $ZnCl_2$) for 70 h at 37°C to allow gelatinase degrade the gelatin *in situ*. The gel was stained with 0.25% Coomassie brilliant blue and destained with 10% acetic acid. Intensity of enzyme reaction bands appeared in the destained gel was quantified by an image analysis using a gel imaging system Printgraph with CS Analyzer software (Atto, Tokyo, Japan).

2.4. *Colorimetric assay of gelatinase activity*

Colorimetric assay of gelatinase activity was performed using a thiopeptolide substrate (Ac-Pro-Leu-Gly-SCH[$CH_2CH(CH_3)_2$]-CO-Leu-Gly-OC_2H_5; Funakoshi, Tokyo, Japan) (*21,22*). FCS was used as a source of gelatinase. Assay was performed using a 96-well microassay plate. Reaction mixture contained 50 µL of 50 mM Hepes-NaOH, pH 7.5, containing 10 mM $CaCl_2$, 0.05% Briji-35, and 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; Wako Pure Chemical Industries), 20 µL of FCS, and 20 µL of sample solution such as toad skin extract, galardin, or bufosteroid or the relevant solvent as control. Enzyme reaction was started by adding 10 µL of 1 mM thiopeptolide substrate dissolved in dimethyl sulfoxide and the mixture was incubated at 37°C. 2-Nitro-5-thiobenzoic acid produced by a reaction of DTNB and sulfhydryl group of cleaved thiopeptolide substrate was continuously detected at 415 nm for 6 h at 1 h intervals using a microplate reader (Model iMark; Bio-Rad Laboratories, Hercules, CA, USA).

2.5. *Fluorometric assay of gelatinase activity*

Fluorometric assay of gelatinase activity was performed using gelatin-fluorescein isothiocyanate (FITC) (Cosmo Bio) as a substrate (*23*). Gelatin-FITC was dissolved in 10 mM acetic acid to a concentration of 1 mg/mL. The developing buffer for gelatin zymography described above was used as a reaction buffer. Reaction mixture was prepared in a shading tube, which contained 70 µL of reaction buffer, 60 µL of MDA-MB-231 cell culture supernatant as a source of gelatinase, and 20 µL of sample solution of interest or the relevant solvent as control. Enzyme reaction was started by adding 50 µL of gelatin-FITC solution and the mixture was incubated for 24 h at 37°C. The reaction was stopped by adding 200 µL of 22%(w/v) trichloroacetic acid and then centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant (300 µL) was mixed with 100 µL of pH adjusting solution, a mixture of 1.5 M Tris-HCl, pH 7.5, and 3.14 M NaOH in the ratio of 11: 21 (v/v). FITC released by the enzyme reaction was measured at 520 nm with excitation at 495 nm using Fluorometer RF-1500 (Shimadzu, Kyoto, Japan).

2.6. *Fractionation of toad skin extract by the Bligh & Dyer method*

Fractionation of water-soluble and $CHCl_3$-soluble components in toad skin extract was performed according to the Bligh & Dyer method (*24*). To 0.9 part of toad skin extract, 1 part each of methanol (MeOH)

and chloroform ($CHCl_3$) were added and vortexed for 10 min followed by a centrifugation for 10 min at 3,000 rpm. The upper layer was moved into another sample vial. The lower layer was extracted again after adding a previously prepared upper layer of $CHCl_3$/ MeOH/ water (1:1:0.9, v/v) and the lower layer was recovered after centrifugation. In contrast, the upper layer of the first extraction was secondary extracted after adding a previously prepared lower layer of $CHCl_3$/ MeOH/ water (1: 1: 0.9, v/v) and the upper layer was recovered after centrifugation. Each layer was dried out *in vacuo* using VaporMix (EYELA, Tokyo, Japan) after removing $CHCl_3$ under N_2 gas. The residues obtained from the upper and lower layer were dissolved in distilled water and MeOH, respectively, of the same volume of toad skin extract sample used.

2.7. *Data analysis*

Data were analyzed by Student's *t* test with a StatMate III software (ATMS, Tokyo, Japan) and a *p* value less than 0.05 was considered significant.

3. Results and Discussion

Gelatinase species in FCS and serum-free culture medium of MDA-MB-231 cells were detected by gelatin zymography. As shown in Figure 1A, a different profile of enzyme reaction bands was shown between FCS and MDA-MB-231 cell culture medium. The bands a-d at positions of molecular weights 92, 80, 72, and 67 kDa, respectively, were deduced as proMMP-9, MMP-9, proMMP-2, and MMP-2, respectively (*20*). Quantification of the enzyme reaction bands by the image analysis showed that the major gelatinase species contained in FCS and MDA-MB-231 cell culture medium are MMP-2 and MMP-9, respectively (Figures 1B and 1C).

Gelatinase activity in FCS in which MMP-2 was a major MMP component (Figure 1B) was assayed using colorimetric assay in the presence or absence of diluents of toad skin extract. As shown in Figure 2A, the enzyme activity was prevented depending on the dilution rate of toad skin extract. In contrast, as shown in Figure 2B, gelatinase activity was not prevented by bufosteroids including bufalin, cinobufagin, and regibufogenin (1 μM each), all of which are known as antitumor ingredients in toad skin extract (*10,25*), while the activity was effectively prevented by 1 μM galardin, a peptide-mimic gelatinase inhibitor (*26*). These results suggest that toad skin extract contains some component which can inhibit gelatinase activity but it is presumably different from bufosteroids.

Next, culture supernatant of MDA-MB-231 cells was used as a gelatinase source in which MMP-9 was a major MMP component (Figure 1C). When gelatinase activity was estimated using FITC-gelatin as a substrate in the presence of 100-fold diluent of toad skin extract, the activity was reduced to 32.9 ± 9.3% compared with untreated control (data not shown). Similarly, in the presence of 10 μM galardin, the residual gelatinase activity was 26.2 ± 4.6% (data not shown).

The enzyme reaction was assayed in the presence of water-soluble or $CHCl_3$-soluble fractions of toad skin extract that were prepared by the Bligh & Dyer method

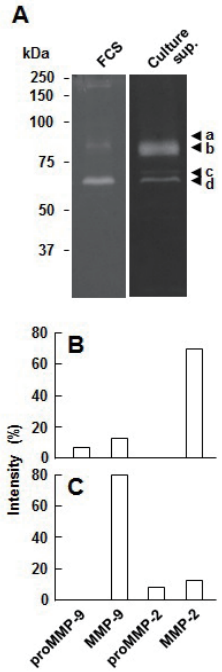

Figure 1. Detection of gelatinases in FCS and culture supernatant of MDA-MB-231 cells using gelatin zymography. A, Gelatin zymography. Arrow heads a-d are deduced to be proMMP-9 (92 kDa), MMP-9 (80 kDa), proMMP-2 (72 kDa), and MMP-2 (67 kDa), respectively. **B** and **C**, Relative intensity of active bands of various MMP species contained in FCS (**B**) and culture supernatant of MDA-MB-231 cells (**C**).

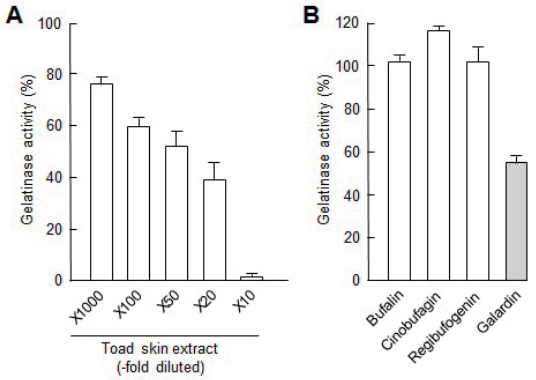

Figure 2. Inhibition of gelatinase activity in FCS by toad skin extract but not by bufosteroids. A, Gelatinase activity was assayed in the presence of various degree of dilution of toad skin extract using colorimetric assay as described in Materials and Methods. **B**, Gelatinase activity was assayed in the presence of 3 types of bufosteroids indicated in the figure or galardin (1 μM each). Data represent percentages compared with untreated control (means ± SD, $n = 4$).

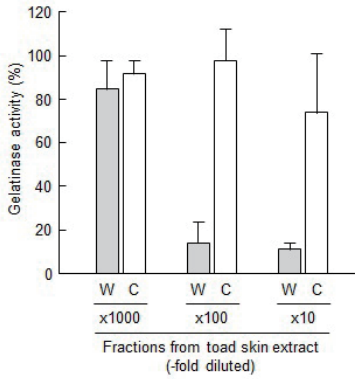

Figure 3. Inhibition of gelatinase activity in culture supernatant of MDA-MB-231 cells by water-soluble fraction but not by $CHCl_3$-soluble fraction of toad skin extract. Toad skin extract was separated to water-soluble (W) and $CHCl_3$-soluble (C) fractions by the Bligh & Dyer method. Gelatinase activity was assayed in the presence of the fractions diluted as indicated using fluorometric assay as described in Materials and Methods. Data represent percentages compared with untreated control (means ± SD, $n = 3$).

as described in Materials and Methods. As shown in Figure 3, gelatinase activity was significantly reduced in the presence of water soluble fraction depending on the dilution rate of the fraction. In contrast, significant reduction was not found in the presence of $CHCl_3$-soluble fraction. This suggests that inhibitory ability of toad skin extract against gelatinases is due to some water-soluble substance.

Major ingredients getting attention in the toad skin extract have been bufosteroids due to their apoptosis-inducing ability to cancer cells (*8,10*). In the present study, bufosteroids such as bufalin, cinobufagin, and regibufogenin at the concentration of 1 μM did not show inhibitory ability against gelatinases (Figure 2B). In addition, $CHCl_3$-soluble lipid fraction prepared by the Bligh & Dyer method did not have apparent ability to prevent gelatinase, although the bufosteroids could be contained in this lipid-rich fraction. Actually, analysis of cell toxicity of water-soluble and $CHCl_3$-soluble fractions using a sulforhodamine B method (*27*) suggested that $CHCl_3$-soluble fraction showed a remarkable toxicity to MDA-MB-231 cells, but water-soluble fraction not (unpublished data).

The present data suggests that toad skin extract have some water-soluble ingredient possessing inhibitory ability to gelatinases. Gelatinases are known to act in the degradation of basement membranes at the time of cancer cell migration into stromal tissues (*1,2*). We have previously found that toad skin extract can suppress the migration of MDA-MB-231 cells not only into a model stromal tissue constituted by type I collagen gels (*16*) but also into a basement membrane model tissue constituted by type IV collagen gels (unpublished data). Therefore, the water-soluble substance in toad skin extract may influence cancer cell migration *via* preventing activity of gelatinases such as MMP-2 and -9.

A lot of compounds that can inhibit MMP activity or expression have been reported, in which some are synthetic compounds and the others are from naturally-occurring substances. Many of synthetic compounds, which mainly categorized into a zinc-binding group and a peptide-mimic group, act as competitive inhibitors against MMP activity (*3,28-31*). In contrast, many of naturally-occurring substances that can control MMP activity have a diversity in the structure including steroids, flavonoids, rotenoids, and terpenoids, and suppress expression of MMP gene by a regulation of signal transduction in cells (*32-36*). Although structural property and action mechanism to prevent gelatinase activity of the substance in toad skin extract have remained unknown, the substance, which is water-soluble and derived from natural product, might have novelty.

In conclusion, our present study suggests that an aqueous extract from toad skin possesses a potent ability to prevent activity of gelatinases such as MMP-2 and -9. Further experiments on the causative substance would improve usability of the toad skin extract cinobufacini to regulate cancer cell migration accelerated by the MMPs.

References

1. Deryugina E. Experimental approaches for understanding the roles of matrix metalloproteinases in cancer invasion. In: Matrix Proteases in Health and Disease (Behrendt N, ed.). Wiley-VCH, Weinheim, Germany, 2012; pp. 181-226.
2. Hagedorn EJ, Sherwood DR. Cell invasion through basement membrane: the anchor cell breaches the barrier. Curr Opin Cell Biol. 2011; 23:589-596.
3. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science. 2002; 295:2387-2392.
4. Augé F, Hornebeck W, Laronze JY. A novel strategy for designing specific gelatinase A inhibitors: potential use to control tumor progression. Crit Rev Oncol Hematol. 2004; 49:277-282.
5. Zhang J, Li X, Zhu H, Wang Q, Feng J, Mou J, Li Y, Fang H, Xu W. Design, synthesis, and primary activity evaluation of pyrrolidine derivatives as matrix metalloproteinase inhibitors. Drug Discov Ther. 2010; 4:5-12.
6. Tauro M, McGuire J, Lynch CC. New approaches to selectively target cancer-associated matrix metalloproteinase activity. Cancer Metastasis Rev. 2014; 33:1043-1057.
7. Lu CX, Nan KJ, Lei Y. Agents from amphibians with anticancer properties. Anticancer Drugs. 2008; 19:931-939.
8. Qi F, Li A, Inagaki Y, Kokudo N, Tamura S, Nakata M, Tang W. Antitumor activity of extracts and compounds from the skin of the toad *Bufo bufo* gargarizans Cantor. Int Immunopharmacol. 2011; 11:342-349.
9. Qi J, Tan CK, Hashimi SM, Zulfiker AH, Good D, Wei MQ. Toad glandular secretions and skin extractions as

anti-inflammatory and anticancer agents. Evid Based Complement Alternat Med. 2014; 2014:312684.
10. Qi FH, Li AY, Lv H, Zhao L, Li JJ, Gao B, Tang W. Apoptosis-inducing effect of cinobufacini, *Bufo bufo* gargarizans Cantor skin extract, on human hepatoma cell line BEL-7402. Drug Discov Ther. 2008; 2:339-343.
11. Wang JY, Chen L, Zheng Z, Wang Q, Guo J, Xu L. Cinobufocini inhibits NF-κB and COX-2 activation induced by TNF-α in lung adenocarcinoma cells. Oncol Rep. 2012; 27:1619-1624.
12. Wang D, Bi Z. Bufalin inhibited the growth of human osteosarcoma MG-63 cells *via* down-regulation of Bcl-2/Bax and triggering of the mitochondrial pathway. Tumour Biol. 2014; 35:4885-4890.
13. Qin TJ, Zhao XH, Yun J, Zhang LX, Ruan ZP, Pan BR. Efficacy and safety of gemcitabine-oxaliplatin combined with huachansu in patients with advanced gallbladder carcinoma. World J Gastroenterol. 2008; 14:5210-5216.
14. Meng Z, Yang P, Shen Y, Bei W, Zhang Y, Ge Y, Newman RA, Cohen L, Liu L, Thornton B, Chang DZ, Liao Z, Kurzrock R. Pilot study of huachansu in patients with hepatocellular carcinoma, nonsmall-cell lung cancer, or pancreatic cancer. Cancer. 2009; 115:5309-5318.
15. Sun T, Zhang Y, Shen Y, Hu K, Zuo M. A case of advanced lung cancer with malignant pericardial effusion treated by intrapericardial Cinobufacini injection instillation. Biosci Trends. 2014; 8:235-239.
16. Nakata M, Mori S, Kamoshida Y, Kawaguchi S, Fujita-Yamaguchi Y, Tang W. Toad skin extract cinobufacini inhibits migration of human breast carcinoma MDA-MB-231 cells into a model stromal tissue. Biosci Trends. 2015; 9:266-269.
17. Fukuda K, Kamoshida Y, Kurokawa T, Yoshida M, Fujita-Yamaguchi Y, Nakata M. Migration of breast cancer cells into reconstituted type I collagen gels assessed *via* a combination of frozen sectioning and azan staining. Biosci Trends. 2014; 8:212-216.
18. Zucker S, Moll UM, Lysik RM, DiMassimo EI, Stetler-Stevenson WG, Liotta LA, Schwedes JW. Extraction of type-IV collagenase/gelatinase from plasma membranes of human cancer cells. Int J Cancer. 1990; 45:1137-1142.
19. Shapiro SD, Fliszar CJ, Broekelmann TJ, Mecham RP, Senior RM, Welgus HG. Activation of the 92-kDa gelatinase by stromelysin and 4-aminophenylmercuric acetate. Differential processing and stabilization of the carboxyl-terminal domain by tissue inhibitor of metalloproteinases (TIMP). J Biol Chem. 1995; 270:6351-6356.
20. Imai K, Okada Y. Purification of matrix metalloproteinases by column chromatography. Nat Protoc. 2008; 3:1111-1124.
21. Weingarten H, Feder J. Spectrophotometric assay for vertebrate collagenase. Anal Biochem. 1985; 147:437-440.
22. Seltzer JL, Weingarten H, Akers KT, Eschbach ML, Grant GA, Eisen AZ. Cleavage specificity of type IV collagenase (gelatinase) from human skin. Use of synthetic peptides as model substrates. J Biol Chem. 1989; 264:19583-19586.
23. Otsuka K, Ohshima M, Kaku M, Kajima T, Fukuoka M, Kaiya Y, Suzuki K. An improved assay method for fibroblast gelatinolytic enzyme. J Nihon Univ Sch Dent. 1997; 39:182-190.
24. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959; 37:911-917.
25. Qi F, Inagaki Y, Gao B, Cui X, Xu H, Kokudo N, Li A, Tang W. Bufalin and cinobufagin induce apoptosis of human hepatocellular carcinoma cells *via* Fas- and mitochondria-mediated pathways. Cancer Sci. 2011; 102:951-958.
26. Galardy RE, Grobelny D, Foellmer HG, Fernandez LA. Inhibition of angiogenesis by the matrix metalloprotease inhibitor *N*-[2R-2-(hydroxamidocarbonymethyl)-4-methylpentanoyl)]-L-tryptophan methylamide. Cancer Res. 1994; 54:4715-4718.
27. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc. 2006; 1:1112-1116.
28. Augé F, Hornebeck W, Decarme M, Laronze JY. Improved gelatinase A selectivity by novel zinc binding groups containing galardin derivatives. Bioorg Med Chem Lett. 2003; 13:1783-1786.
29. Gialeli C, Theocharis AD, Karamanos NK. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. FEBS J. 2011; 278:16-27.
30. Fields GB. New strategies for targeting matrix metalloproteinases. Matrix Biol. 2015; 44-46:239-246.
31. Vandenbroucke RE, Libert C. Is there new hope for therapeutic matrix metalloproteinase inhibition? Nat Rev Drug Discov. 2014; 13:904-927.
32. Lee HS, Seo EY, Kang NE, Kim WK. [6]-Gingerol inhibits metastasis of MDA-MB-231 human breast cancer cells. J Nutr Biochem. 2008; 19:313-319.
33. Yoon JH, Choi YJ, Lee SG. Ginsenoside Rh1 suppresses matrix metalloproteinase-1 expression through inhibition of activator protein-1 and mitogen-activated protein kinase signaling pathway in human hepatocellular carcinoma cells. Eur J Pharmacol. 2012; 679:24-33.
34. Xia JF, Gao JJ, Inagaki Y, Kokudo N, Nakata M, Tang W. Flavonoids as potential anti-hepatocellular carcinoma agents: recent approaches using HepG2 cell line. Drug Discov Ther. 2013; 7:1-8.
35. Liang Y, Huang M, Li J, Sun X, Jiang X, Li L, Ke Y. Curcumin inhibits vasculogenic mimicry through the downregulation of erythropoietin-producing hepatocellular carcinoma-A2, phosphoinositide 3-kinase and matrix metalloproteinase-2. Oncol Lett. 2014; 8:1849-1855.
36. Shang HS, Chang JB, Lin JH, Lin JP, Hsu SC, Liu CM, Liu JY, Wu PP, Lu HF, Au MK, Chung JG. Deguelin inhibits the migration and invasion of U-2 OS human osteosarcoma cells *via* the inhibition of matrix metalloproteinase-2/-9 *in vitro*. Molecules. 2014; 19:16588-16608.

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Case Report

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Hepatic venous outflow block caused by compressive fecaloma in a schizophrenic patient treated with clozapine

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Summary **In Clozapine users constipation is among the reported side effects including agranulocytosis and myocarditis with prevalence rates ranging from 14% to 60%. In extreme cases this may lead to bowel obstruction and paralytic ileus which, if not detected and treated early, may lead to mortality up to 30%. We report the first case of hepatic outflow block secondary to compression of the liver by a distended colon upstream an impacted fecaloma in a 47-year old schizophrenic man treated by clozapine. Emergency sub-total colectomy was performed for pan-colonic ischemia. Surgery relieved the liver outflow block and was followed by uneventful outcome. Patients receiving clozapine should undergo routine laxatives and monitoring in order to limit the risk of clozapine-related ileus and bowel ischemia.**

Keywords: Fecaloma, hepatic venous outflow obstruction, liver congestion

1. Introduction

Schizophrenia affects approximately 26 million people worldwide (*1*).Clozapine is one of the most effective antipsychotic drugs in refractory schizophrenia. In clozapine users constipation is among the most common side effects including agranulocytosis and myocarditis with prevalence ranging from 14% to 60% (*2*). In extreme cases this may lead to bowel obstruction and paralytic ileus which, if not detected and treated early may lead to mortality up to 30% of cases (*3*). We report here the first case of liver venous outflow block due to compression by a distended colon upstream impacted fecaloma in a schizophrenic patient treated with clozapine.

2. Case report

A 47-year-old schizophrenic man with schizophrenia, chronically institutionalized and treated with clozapine was seen at our emergency department for severe abdominal pain. He had a blood pressure of 90/60 millimeter of mercury (mm Hg) and appeared to be moderately dehydrated. He had suffered from constipation over the previous 10 years and had been on a high-fiber diet and laxatives. The patient was not under any other medication according to the accompanying nurse and he did not have any surgical history. At physical examination, the following parameters were noted: hypothermia (35.7°C), decreased blood pressure (90/60 mm Hg), and mild deshydratation. Abdominal examination revealed severe and diffuse tenderness with muscle guarding. Impacted hard fecaloma deemed impossible to extract was found in the rectal vault at rectal examination. In the emergency room, he developed sudden feculent vomitus and inhalation. Following immediate resuscitation including respiratory intubation, abdominal computed tomography revealed largely dilated colon measuring 11 cm in diameter with fecal impaction. The liver was lifted upwards and compressed by the distended colon with subsequent Budd-Chiari syndrome: the inferior vena cava was compressed and the hepatic veins were poorly contrasted (Figure 1). Laboratory explorations showed liver insufficiency (prothrombin time = 55%, and factor V = 46%), cytolysis (aspartate aminotransferase = 141 International Unit/Liter (IU/L)

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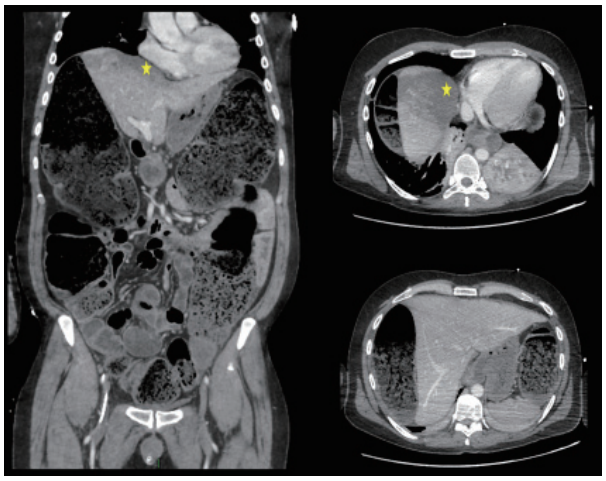

Figure 1. Computed tomography revealed the presence of a giant fecaloma causing hepatic venous outflow obstruction by extrinsic compression (yellow star indicates the areas of liver congestion).

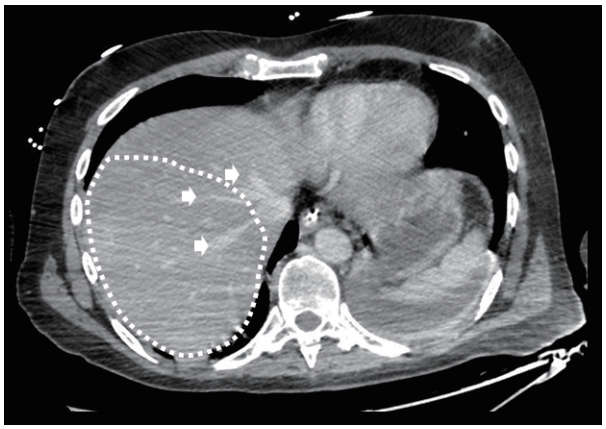

Figure 2. Computed tomography revealed that hepatic veins were patent and continuous after subtotal colectomy (white stars indicate the hepatic veins). The right liver, invisible on preoperative computed tomography becomes visible following decompression (white small lines).

(normal < 45 IU/L) and alanine aminotransferase = 104 IU/L (normal < 45 IU/L)) and leukocytosis (white blood cell count = 25,000/mm^3). Emergency laparotomy was indicated for both peritonitis and mechanical acute Budd-Chiari syndrome. Subtotal colectomy for pan-colonic ischemia was performed with double-end ileo-sigmoidostomy together with manual extraction of the remainder of the fecaloma from the sigmoid and rectum. The specimen examination showed lesions compatible with idiopathic megacolon including atrophy of the mucosa and submucosa and several foci of necrosis, and ruled out melanosis coli. The postoperative course was uneventful, liver function tests returned to normal values, and liver imaging normalized (Figure 2). The patient was transferred day 21 to his psychiatric center. Digestive continuity was restored 3 months and the patient is currently treated with clozapine with a satisfactory bowel transit.

3. Discussion

To the best of our knowledge, this is the first case of severe clozapine-related constipation with massive colonic distension causing hepatic venous outflow block. As well it is the first case of Budd-Chiari syndrome with such mechanism (*4*). Constipation is among the most frequent side-effects antipsychotic therapy and ranks in the top 4 of clozapine with a prevalence ranging from 5% to 60% (*2*). Severe and even fatal complications of constipation have been reported in this setting. The most common causes of death include severe fecal impaction leading to bowel necrosis, sepsis, colonic perforation, and inhalation of feculent vomitus (*3*). Late diagnosis of these complications is due to the combination of an increased pain threshold (related to both the mental disorder and the adverse effects of treatment), and indifference and problems with adequate expressions of pain sensations.
In conclusion, despite adequate diet and laxative prescription, constipation remains a serious side-effect of clozapine. Ongoing monitoring for constipation should be meticulously performed with each prescription and refill for clozapine.

References

1. World Health Organization. The global burden of disease: 2004 update. *http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/* (accessed May 22, 2014).
2. De Hert M, Dockx L, Bernagie C, Peuskens B, Sweers K, Leucht S, Tack J, Van de Straete S, WAmpers M, Peuskens J. Prevalence and severity of antipsychotic related constipation in patients with schizophrenia: a retrospective descriptive study. BMC Gastroenterol. 2011; 11:17.
3. Hibbard KR, Propst A, Frank DE, Wyse J. Fatalities associated with clozapine-related constipation and bowel obstruction: A literature review and two case reports. Psychosomatics. 2009; 50:416-419.
4. Goel RM, Johnston EL, Patel KV, Wong T. Budd-Chiari syndrome: investigation, treatment and outcomes. Postgrad Med J. 2015; 91:692-697.

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Subject Index (2015)

Editorial

Reviews

Original Articles

Nwibo DD, Matsumoto Y, Sekimizu K
2015; 9(3):184-190. (DOI: 10.5582/ddt.2015.01036)

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Shen YZ, Zhang LJ, Jia XF, Zhang YX, Lu HZ
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Association between maternal diabetes mellitus and the risk of congenital malformations: A meta-analysis of cohort studies.
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2015; 9(4):274-281. (DOI: 10.5582/ddt.2015.01044)

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Brief Reports

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