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Editorial Board

Editorial and Head Office:

Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan
E-mail: office@ddtjournal.com

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Drug substitution and adjuvant therapy in patients with genetics related infertility: A review

Jun Zhu[1,§], Jiaxi Jin[1,§], Qing Qi[2,3,4], Qi Zhou[5], Jing Zhou[2,3,4,*], Ling Wang[2,3,4,*]

[1] The Affiliated Wenling Hospital of Wenzhou Medical University, Zhejiang, China;
[2] Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China;
[3] The Academy of Integrative Medicine of Fudan University, Shanghai, China;
[4] Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, Shanghai, China;
[5] Department of Gynecology, Traditional Chinese Medicine Hospital of Yangpu District, Shanghai, China.

SUMMARY With the in-depth study of the human genome and the increasing popularity of gene sequencing, it has been gradually confirmed that genetics can play a crucial role in infertility. To provide references for clinical treatment, we have focused on genes and drug therapy for genetic infertility. This review recommends adjuvant therapy and drug substitution. Examples of these therapies include antioxidants (such as folic acid, vitamin D, vitamin E, inositol, coenzyme Q10 *etc.*), metformin, anticoagulants, levothyroxine, dehydroepiandrosterone, glucocorticoids, and gonadotropins *etc.* Based on the pathogenesis, we provide an overview of the current knowledge, including randomized controlled trials and systematic reviews, and predict potential target genes and signaling pathways, proposing possible future strategies for the use of targeted drugs to treat infertility. Non-coding RNAs are anticipated to become a novel target for the treatment of reproductive illnesses since they have a significant role in controlling the occurrence and development of reproductive diseases.

Keywords Genetics, infertility, drug substitution, adjuvant therapy, targeted drugs

1. Infertility overview

Infertility, a state of subfertility, refers to the inability to establish a clinical pregnancy after 12 months of regular unprotected sexual intercourse with a healthy partner. Unfortunately, 15% of newlywed couples struggle with infertility. The cause of infertility is specifically associated with females 25%-35% of the time, males 30% of the time, co-contributing factors 25%-40% of the time, and unexplained or idiopathic causes 10%-20% of the time (*1*). Infertility is a complex disease caused by genetics, anatomical abnormalities, immunological abnormalities, endocrine problems, and the environment, with genetics being one of the main areas of study regarding this topic.

2. Literature on infertility and genetics

With the in-depth study of the human genome and the increasing popularity of gene sequencing, it has been gradually confirmed that genetics can play a crucial role in infertility. Clinical phenotypes are complicated and dynamic, and infertility diagnosis and treatment frequently include interdisciplinary approaches. To provide references for clinical treatment, we focus on genes connected to infertility.

2.1. Female

Infertility can arise from abnormalities in any of the processes that control ovarian growth, oocyte maturation, fertilization ability, and early embryonic developmental in females. An increasing number of mutations in genes (such as *FSHR*, *mT-TRNA*, *PATL2*, *TUBB8*, *TRIP13*, *CDC20*, *ZP*, *TLE6*, *WEE2*, *BTG4*, *PANX1*, *PADI6*, *NLRP2*, *NLRP5*, *KHDC3L*, *REC114* and *MTHFR*) have been proven to be pathogenic causes of endometrial receptivity deficiency, oocyte maturation arrest, fertilization disorder and early embryo arrest (*2*).

Uterine dysfunction can prevent embryo implantation. Aside from abnormal uterine structure, the endometrium seems to be an important factor in implantation failure and later stages of pregnancy, including placentation, foetal development, pregnancy outcome and postnatal health after implantation. Importantly, it has been reported that many genetic factors are implicated in endometrial receptivity. During the implantation window in Chinese patients with polycystic ovary syndrome

(PCOS), TM4SF4 and MMP26 are particularly downregulated, indicating that differential endometrial gene expression contributes to endometrial receptivity dysfunction (*3*). In addition, the *ICAM-1* gene and *MUC1* gene play crucial roles in cellular adhesion in the endometrium, however, it has been proven that genetic polymorphism in the *ICAM-1* and *MUC1* domain may be proven that genetic polymorphisms in the *ICAM-1* and *MUC1* domains may be associated with susceptibility to endometriosis, leading to implantation failure or endometriosis, recurrence (*4*). In addition, the expression of other altered genes, including those encoding MME and WWP1, were greatly enhanced, whereas the expression of the gene encoding TNC, LIF and HOXA-10 were decreased in recurrent implantation failure samples during the implantation window (*5,6*). Collectively, these altered genes may serve as targets for improving endometrial receptivity.

2.1.1. Oocyte maturation arrest

Human oocytes can be fertilized through three stages: the germinal vesicle (GV), metaphase I (MI) stage and metaphase II (MII) stage. The genetic origin of oocyte maturation arrest, a major kind of infertility characterized by the production of immature oocytes, is yet unknown. In certain close families with a GV-blocking phenotype, *PATL2* mutations have been identified that lead to improper oocyte maturation. PATL2 deficiency disrupted oocyte maturation, according to a study by Marie *et al.* (*7*) utilizing *PALT2* knockout mice. Additionally, *TUBB8* mutations disrupted oocyte meiotic spindle assembly, oocyte maturation and microtubule behaviour, causing MI arrest in oocytes and female infertility (*8*). Another theory links a *TRIP13* mutation to female infertility characterized by oocyte meiotic arrest (*9*). The primary downstream target of spindle assembly checkpoint inhibition is the mitotic activator CDC20. As a result, suppression of CDC20 could impede sister chromatin separation, resulting in female infertility due to oocyte maturation arrest, fertilization problems, and early embryo arrest (*9*).

2.1.2. Fertilization disorder

To our knowledge, the zona pellucida (ZP) is crucial to the development of the preimplantation embryo and to other reproductive processes. There are four glycoproteins in Z*P* (ZP1, ZP2, ZP3 and ZP4). Infertility caused by abnormal ZP-free oocytes, such as oocyte degeneration and empty follicle syndrome, is caused by mutations in human genes *ZP1*, *ZP2* and *ZP3* (*2*). WEE2 is a crucial meiotic regulator. Numerous *WEE2* mutations have recently been found to be responsible for failed human fertilization. Reduced WEE2 protein levels or cellular compartment relocation caused by mutated *WEE2* in HeLa cells resulted in lower levels of phosphorylated cell division control protein 2 homologue and failed fertilization (*10*).

2.1.3. Early embryo arrest

Several mutations in the subcortical maternal complex genes, including *FLOPED*, *PADI6*, *NLRP2*, *TLE6* and *NLRP5*, have been linked to early embryo arrest-related female infertility (*11*). A specific early embryonic phenotype known as zygotic cleavage failure is characterized by normal oocytes that could be successfully fertilized but were unable to complete the first cleavage. The connection of CNOT7 and eIF4E, two essential translation initiation factors, and BTG4, a crucial adaptor of carbon catabolite repression 4-negative on tata-less, promotes the degradation of maternal mRNAs (*12*). The eggs of *BTG4*-null females can be fertilized successfully but fail to be cleaved. Four homozygous mutations of *BTG4* (c.73C>T (p.Gln25Ter), c.1A>G (p.?), and c.475_478del (p.Ile159LeufsTer15), c.166G>A (p.Ala56Thr)) are related to the phenotype of zygotic cleavage failure (*12*). Mutations in CDC20 have also been discovered to cause early embryonic arrest (*12*). It has previously been demonstrated that early embryonic arrest results from mutations in *REC114* and *KHDC3L* (*13*).

2.2. Male

Spermatogenesis is a multistep process that includes three primary stages: spermatogonia growth, spermatocyte meiosis, and mature sperm cell differentiation following meiosis. Azoospermia, oligospermia, asthenospermia, and teratospermia are a few examples of poor semen quality that are associated with male infertility. Idiopathic male infertility may be caused by a variety of factors, including abnormal genes and sperm mitochondria, the impact of environmental contaminants, and abnormal hormone metabolism.

2.2.1. Azoospermia and oligospermia

The total lack of spermatozoa in ejaculated semen is known as azoospermia. Sperm density < 15 million/mL is known as oligospermia. As a rule, chromosomal abnormalities, Y chromosome microdeletions in the azoospermia factor region, and mutations in the cystic fibrosis gene are the most prevalent genetic abnormalities. Azoospermia can be separated into obstructive azoospermia and nonobstructive azoospermia. Congenital bilateral absence of the vas deferens (CBAVD) is the most common cause of obstructive azoospermia; 68%-80% of cases of CBAVD are caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (*14*). In addition, follicle-stimulating hormone (FSH) signaling may be regulated by polymorphisms of *FSHB* genes (rs1394205, c.-29G

>A; rs6165, c.919A>G; rs6166, c.2039 A>G) (*15*) and *FSHR* genes (rs10835638, c.-211G>T) (*16*), which are always associated with azoospermia.

The most prevalent hereditary cause of male infertility, Creutzfeldt-Jakob syndrome, is characterized by severe oligozoospermia or azoospermia (> 90%) (*17*). Creutzfeldt-Jakob syndrome patients may experience infertility as a result of spermatogenesis issues, including sex hormone imbalance, serological cell failure, and spermatogonial stem cell loss. Additionally, the congenital absence of the vas deferens, idiopathic hypogonadotropic hypogonadism, isolated hypogonadotropic hypogonadism, androgen insensitivity syndrome, immotile cilia syndrome, and round head spermatozoa are genetic mutations linked to male infertility caused by Creutzfeldt-Jakob syndrome (*17,18*).

A significant contributing factor to azoospermia or severe oligozoospermia is Y chromosome microdeletion. Its prevalence is second only to that of Creutzfeldt-Jakob disease (*18*). The term "Y chromosome microdeletion" refers to the loss of azoospermia components on the long arm of the Y chromophobe; this deletion accounts for 60%-70% of all cases. Clinical symptoms caused by Y chromosomal microdeletions are diverse, resulting in phenotypes that range from azoospermia to normal sperm count but abnormal morphology (*18*).

2.2.2. Asthenozoospermia

Males with asthenozoospermia have sperm motility problems, such as reduced or absent motility that worsens over time. Numerous genetic variables, including as chromosomal mutations, sperm motility-related gene mutations, mtDNA abnormalities, epigenetic aberrations, and micro RNAs (miRNAs), contribute to the aetiology of asthenozoospermia (*19*).

2.2.3. Teratozoospermia

The abnormal morphology of more than 85% of sperm cells following ejaculation is referred to as teratozoospermia. Sperm cells with abnormal morphology are influenced by genetic factors such as aneuploidy, sperm DNA breaks, and mutations. It is crucial for gamete fusion, sperm flagellum movement, and the acrosome reaction to be regulated by the protein CRISP2, which is found in sperm. Asthenoteratozoospermic males exhibit low levels of CRISP2 protein expression in their ejaculated sperm, which is associated with reduced sperm motility, abnormal morphology, and infertility (*20*).

2.3. Genetic disorders associated with infertility

2.3.1. *MTHFR* genetic polymorphism-induced infertility

A number of enzymes are needed to catalyse the metabolic activity of folic acid in the body. Folic acid is an essential B vitamin that is involved in the production of purine and pyrimidine and is crucial for protein synthesis and cell division. In the metabolism of folic acid, methylenetetrahydrofolate reductase (MTHFR) is an essential enzyme. Homocysteine (Hcy) accumulation (*21*) and DNA hypomethylation (*22*) are caused by incorrect catalytic steps of gene-related enzymes and low folic acid levels caused by the *MTHFR* gene mutation.

Hcy results in poor villui vascularization and hypercoagulability by destroying vascular endothelial cells. More research revealed that the thrombogenic effect of Hcy was predominantly closely related to Hcy harming vascular endothelial cells, inducing the formation of thromboxane and prostaglandin through thiolactone, thereby encouraging platelet aggregation, increasing the activity of coagulation Factor V, and inhibiting the fibrinolytic system. Therefore, Hcy affects embryo implantation by decreasing endometrial receptivity (*23*).

Reactive oxygen species (ROS) synthesis and removal in semen are dynamically balanced under physiological conditions, and the right amount of ROS is necessary for various physiological sperm functions, including energy acquisition and the acrosomal response. When the dynamic equilibrium is disturbed, an excess of ROS causes oxidative damage to proteins, DNA, and sperm membranes. Increased ROS can also influence the expression of associated genes, which in turn modify the quantity of specific chemicals in semen. Increased ROS can also disrupt various signaling pathways in cells. Recent research has indicated that variations in the *MTHFR* gene, particularly at the polymorphic loci of *MTHFR* C677T, and A1298C, are linked to male infertility (*24*). Folic acid deficiency lowered MTHFR activity and led to hyperhomocysteinaemia, which disrupted ROS homeostasis in semen and caused DNA damage to sperm (*25*). Additionally, hyperhomocysteinemia is significantly associated with disease severity and is closely related to cardiovascular and cerebrovascular diseases (*26*). Atherosclerosis in the arteries of the testis will disrupt spermatogenesis if there is insufficient blood flow (Figure 1).

Non-coding RNAs (NcRNAs) play pivotal roles in the aetiology of diseases, including those associated with hyperhomocysteinaemia. A previous study confirmed that miRNAs played essential roles in the regulation of gene expression and certain transitions between the main phases of spermatogenesis in mammals (*27*). The *MTHFR* 3'-UTR rs55763075 polymorphism was associated with folate and Hcy in men with idiopathic azoospermia, which may modify the susceptibility to male infertility (*28*).

2.3.2. Premature ovarian insufficiency

Premature ovarian insufficiency (POI) refers to

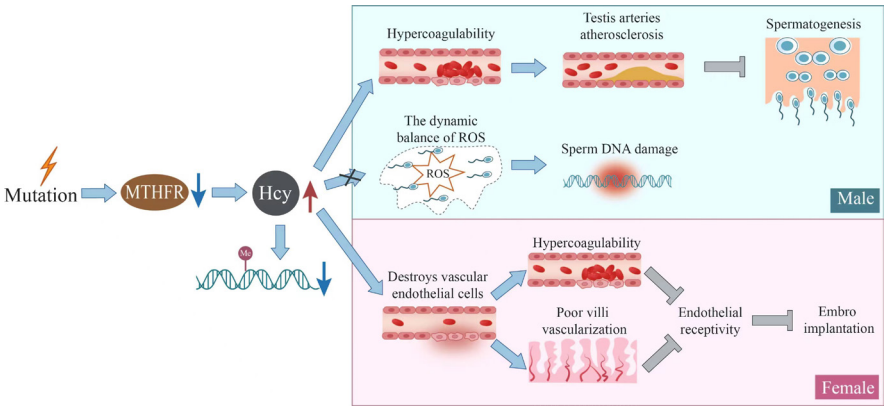

Figure 1. Mutation in *MTHFR* gene decreases MTHFR, resulting in Hcy and DNA hypomethylation. For female, Hcy destroys vascular endothelial cells, leading to poor villi vascularization and hypercoagulability. Therefore, Hcy results in reducing endometrial receptivity and influencing embryo implantation. For male, Hcy destroys the dynamic balance of ROS in semen, leading to sperm DNA damage. In addition, hypercoagulability caused by Hcy may lead to atherosclerosis in the arteries of testis, affecting spermatogenesis.

the occurrence of ovarian dysfunction in women before the age of 40 years. Patients with POI have significantly reduced or lost fertility due to severe ovulatory dysfunction. This condition has a solid genetic foundation. Genetic factors include mitochondrial dysfunction, X chromosome abnormalities, gene mutation, and other elements. Up to 94% of POI cases are caused by pathogenic chromosomal abnormalities. One of the significant genetic causes of POI is gene mutation. Mitochondrial DNA (MtDNA) is closely related to the quality of oocytes, and a decrease in mtDNA copy number in oocytes leads to a decrease in mitochondrial number, oocyte apoptosis and arrest. The mtDNA of oocytes is more susceptible to assault by ROS than that of other cells because of the low level of oxidative phosphorylation and absence of histone protection. Particularly when there is oxidative stress, the likelihood of mtDNA deletion or mutation will be considerably increased (*3*). Current studies have found *mT-TRNA* mutations in POI patients, such as MFN2, DNM1L, C3303T, A4435G, T4363C, G5821A and A15951G (*29,30*).

Increasing evidence has demonstrated that circular RNAs (circRNAs) are dysregulated in many diseases, such as cancer, cardiovascular diseases and neurological diseases. Moreover, a recent study identified 133 upregulated and 424 downregulated circRNAs in patients with POI, suggesting that differentially expressed circRNAs in the pathogenesis of POI (*31*). Additionally, miRNAs also participate in the development of POI. Numerous miRNAs including miR-23a, miR-27a, miR-22-3p, miR-146a, miR-196a, miR-290-295, miR-423, and miR-608 are significantly associated with POI (*32*). MiRNA-379-5p is implicated in the pathophysiology of biochemical POI by modulating PARP1 and XRCC6 (*33*). Therefore, dysregulated circRNAs and miRNAs are imperative in the development and progression of POI in women.

2.3.3. Hereditary prethrombotic state

A class of hereditary disorders known as hereditary prethrombotic states is predisposed to thrombosis and a hypercoagulable state of blood as a result of numerous genetic abnormalities. The development of follicle blood vessels, known as angiogenesis, is known to play a significant role in both the follicular and luteal stages. Ovarian function may be affected by genes related to thrombosis and vascular homeostasis, and enhanced Factor v activation brought on by *Factor v leiden* mutations may result in ovarian microthrombosis (*34*), which in turn alters the vascularization of the follicular system. Once insufficiently vascularized, the follicular microenvironment experiences a decrease in oxygen levels that eventually causes the oocyte/follicular pool to shrink more quickly, which results in the depletion of the follicular/follicular reserve (*35*). Additionally, the prethrombotic state, which impairs microcirculation and lowers patients' endometrial receptivity, is one of the risk factors for repeated implantation failure (*23*).

2.3.4. Obesity

The *leptin* gene produces the adipokine leptin. Patients with obesity may develop insulin resistance (IR) and hyperinsulinaemia as a result of excessive fat buildup. The body is in a chronic inflammatory state as a result of the increased fat release of pro-inflammatory and decreased anti-inflammatory substances in obese patients, which ultimately affects the quality and function of the oocyte. Additionally, a study discovered that obese women had significantly higher levels of oxidized low density lipoprotein, which could raise the body's level of ROS by interacting with the appropriate receptors (*36*). As a result, an oxidative stress response occurred, causing an increase in granulosa cell apoptosis in follicular cells and lowering reproductive potential.

A large body of studies report that numerous miRNAs, circRNAs and long non-coding RNAs (lncRNAs) contribute to the regulation of obesity-related metabolic pathways. For example, obese individuals

exhibit decreased levels of lncRNAs such as Mist, lincIRS2, lncRNA-p5549, H19, GAS5 and SNHG9 in adipose tissues or other biological samples (*37*). Abnormal levels of miRNA-192, miRNA-122, and miRNA-221 were observed in obesity, diabetes, and cardiovascular diseases (CVD), which increase the risk of development of diabetes and MetS and its progression to CVD in patients with obesity (*38*).

2.3.5. Autoimmune thyroid diseases

According to modern medicine, other elements that contribute to the aetiology of autoimmune thyroid disorders (AITDs) primarily include genetic, immunological, dietary, and apoptotic factors. Since there is clear family aggregation in the aetiology of AITDs, genetic factors likely play a significant role in the disease's pathogenesis and may be linked to a few particularly susceptible genes. Several susceptibility genes for AITDs, including *CTLA-4, CD40*, and *HLA-DR*, have been discovered (*39*). Meanwhile, various miRNAs (such as miR-154, miR-376b, miR-22, miR-183, miR-101, miR-197, and miR-660), lncRNAs (IFNG-AS1 and SAS-ZFAT), and circ 0089172 have been identified to be dysregulated in T-cell metabolic reprogramming during AITDs (*40*).

Endometrium, ovulation, and fertility are all negatively impacted by thyroid dysfunction from implantation to birthing. However, uncertainty surrounds the proposed pathogenic mechanism.

2.3.6. Congenital adrenal hyperplasia

CYP21A2 mutation, which results in steroid 21-hydroxylase production problems, is the main cause of congenital adrenal hyperplasia (CAH), a set of autosomal recessive genetic illnesses. Reduced corticosteroid 21-hydroxylase triggers compensatory increases in pituitary and hypothalamic corticotropin-releasing hormone release, which ultimately results in increased androgen and progesterone levels (*41*). The primary cause of the infertility problems experienced by 10%-30% of CAH women of reproductive age is testosterone excess-associated anovulatory cycles (*42*). Through androgen receptors, androgens may directly influence follicular development, while aromatase expression and oestrogen aromatization indirectly influence follicular development (*42*). The viability of fallopian tubes, endometrial receptivity, and cervical thickness are also impacted by higher progesterone levels, which have an impact on embryo implantation (*43*).

3. Drug substitution and adjuvant therapy

A significant societal issue and ongoing medical concern is infertility. Currently, fertility preservation and assisted reproductive technology (ART) are the main treatments for infertility caused by genetics. These methods have been successfully employed to treat infertility in recent years. Despite impressive advancements in infertility, ART failure rates remain high. Additionally, the consequences of ovarian stimulation and accompanying adverse effects are relatively unclear to clinicians. Numerous natural substances have been shown to reduce infertility in a variety of ways. In this review, adjuvant therapy and drug substitution are advised. Examples of these therapies include antioxidants (such as folic acid, vitamin D, vitamin E, inositol, coenzyme Q10 *etc.*), metformin, anticoagulants, levothyroxine (LT4), dehydroepiandrosterone (DHEA), glucocorticoids, gonadotropins *etc.* (Figure 2). The use of these medications in the field of assisted reproduction is still in the exploratory phase. On the basis of the literature, this article reviews the use of medications in infertility.

3.1. Antioxidants

Utilizing oxidant/activating chemicals is one of the treatments for infertility as the importance of oxidative stress energy release in the pathophysiology of infertility is coming to treatment. One way is that the right nutrients can enhance ovarian function. However, optimal supplements may also contain elements that affect the levels of plasma testosterone, activating agents, and antioxidants that increase sperm motility.

3.1.1. Folic acid

Folic acid is an antioxidant whose metabolic route is crucial for nucleotide anabolism, DNA methylation and repair, and the preservation of genomic stability. The treatment of infertility is highly contested. Preovulation folic acid supplementation decreased the rate of embryonic retardation, and it boosted glutathione production in oocytes in animal models where preconception folic acid deficiency impeded ovulation and increased the number of vestigial follicles. This suggests that folic acid is crucial for the growth of follicles. Folic acid is used to support reproductive health through affordable and efficient techniques, and how it is used is influenced by its genetic polymorphisms. Studies have demonstrated that folic acid produced metabolic disorders in women with *MTHFR* gene mutations; nevertheless, even when given in the same amount, folic acid levels in serum and red blood cells were lower than those of healthy individuals (*44*). Recently, Ye *et al.* (*37*) found that *MTHFR* polymorphism rs1801133 was not related to the pregnancy rate or pregnancy outcomes of women undergoing *in vitro* fertilization/intracytoplasmic sperm injection-embryo transfer with adequate synthetic folic acid supplementation by analysing 692 women undergoing *in vitro* fertilization/intracytoplasmic sperm injection-embryo transfer and taking adequate folic

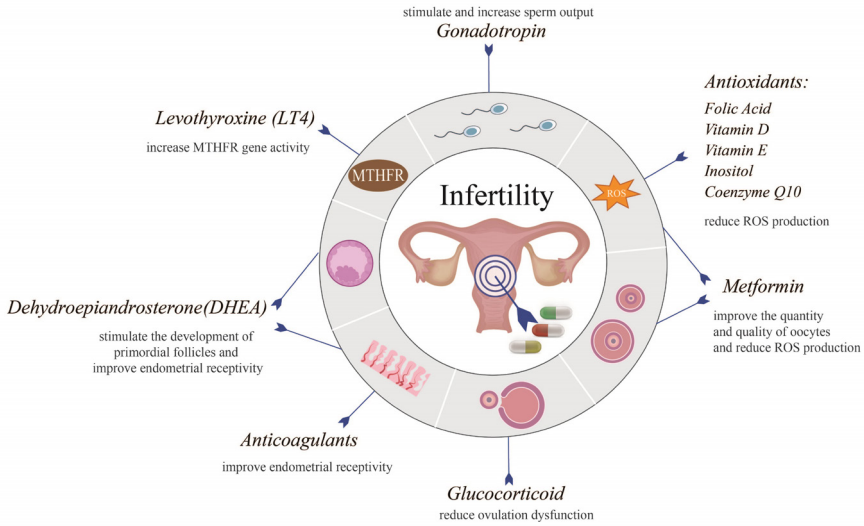

Figure 2. Adjuvant therapy and drug substitution are advised. Examples of these therapies include antioxidants (such as folic acid, vitamin D, vitamin E, inositol, coenzyme Q10 *etc.*), metformin, anticoagulants, levothyroxine, dehydroepiandrosterone, glucocorticoids, and gonadotropins *etc.*

acid. The United States Preventive Services Task Force advises that all women take a multivitamin containing folic acid at least one month before to becoming pregnant to boost a woman's chances of getting pregnant due to the safety of folic acid supplementation (*45*).

Folic acid may enhance sperm characteristics and increase fertility. In oligozoospermia with the *MTHFR* 677TT genotype, folic acid treatment improved semen characteristics, semen malondialdehyde, sperm DNA fragmentation, and pregnancy outcome (*46*). However, several research have claimed that folic acid is unable to enhance the DNA integrity or semen characteristics of male infertility (*47*).

3.1.2. Vitamin D

The biological purpose of vitamin D in the body is to maintain calcium homeostasis, which has an impact on cell differentiation and death. All of these tasks are predominantly carried out by vitamin D receptors, which have been found in some reproductive and endocrine organs including the ovaries, uterus, testes, hypothalamus, and pituitary gland in addition to calcium-regulated tissues (*48*). There are vitamin D receptors in the endometrium. According to a study by Karine *et al.* (*49*), the endometrium becomes thinner as vitamin D levels rise. Supplementing with vitamin D may increase the success rates of *in vitro* fertilization in infertile women (*50*). Only a small percentage of follicles mature into sinus follicles and ovulate at this time, whereas the majority spontaneously degenerate into atresia. Studies have showed a connection between vitamin D administration and the quantity of sinus follicles (*48*).

It is the term used to describe the diminished sensitivity of peripheral tissues to insulin, which reduces the biological impact of insulin. Insufficient use of glucose causes high blood glucose levels, which in turn trigger the pancreatic B cells to secrete more insulin as a balancing mechanism, causing hyperinsulinaemia and infertility. Numerous randomized controlled trials (*51*) have demonstrated that vitamin D administration can lower IR and blood glucose levels, although the precise mechanism relating vitamin D and IR is still unknown. According to some studies (*52*) the mechanism of vitamin D's impact on insulin metabolism may involve directly or indirectly stimulating IR by increasing the expression of the vitamin D receptor, improving insulin's sensitivity to transporting glucose, and reducing the release of pro-inflammatory cytokines. To fully realize the protective action of islet B cells, vitamin D can block their death (*53*) .

In patients with asthenospermia infertility, vitamin D administration can increase the mean sperm concentration and sperm motility as well as the overall clinical pregnancy rate. In fact, some authors claimed that vitamin D treatment had positive benefits on patients' progressing sperm motility and morphology, while others did not (*54*). Other research revealed a connection between the quality, motility, and functionality of mature sperm and the enzymes in charge of vitamin D metabolism in the human sperm flagellum (*55*).

Supplementation with vitamin D could improve the function of endothelial cells in in type 2 diabetes by affecting the expression profiles of 1,791 mRNAs, 2,726 lncRNAs, 205 circRNAs, and 45 miRNAs, and these differentially expressed RNAs were related to matrix metalloproteinase and guanosine triphosphatase activities, specific signaling pathways, and components of actin, extracellular matrix, or adherens junctions (*56*). However, the role of these natural products, nutrients, and supplements on ncRNAs requires further study in infertility.

3.1.3. Vitamin E

An essential antioxidant molecule known as vitamin E (-tocopherol) is thought to stop lipid peroxidation and increase the action of several free radical-scavenging antioxidants. According to Bahadori *et al*. (*57*), vitamin E levels were associated with both the frequency of high-quality embryos and the maturation rate of oocytes. The maturation rate of oocytes with 0.35-1 mg/dL and 1.5-2 mg/dL vitamin E in follicular fluid was greater, while the rate of high-quality embryos with 10-15 mg/dL vitamin E in serum was higher. The outcomes agree with those of Ashraf *et al.* (*58*). Experiments have showed that vitamin E protects sperm from oxidative damage, and improves sperm performance, and is effective in idiopathic infertility (*59*). However, some studies have suggested that vitamin E does not improve semen parameters (*47*).

3.1.4. Inositol

Mammalian oocyte synthesis of intracellular signals, the completion of poly spermatozoa meiosis, and embryo development all depend on inositol. Myo-inositol (MI), D-chiro-inositol, L-chiro-inositol, and shar-inositol are the four isomers that have currently been identified. In clinical settings, inositol drugs mostly refer to MI, which has properties that improve insulin sensitivity as well as anti-oxidation and anti-inflammatory properties. Researchers inquired whether inositol may enhance the quality of eggs and discovered that the levels of MI in follicular fluid were positively associated with those of oestradiol and the quality of the embryos, indicating that high concentrations of MI may be involved in follicular maturation.

Due to its ability to regulate hormones and its antioxidant properties, inositol has been shown to have positive impacts on mitochondrial function and sperm motility. Antioxidant therapy is currently a common treatment option for oligospermia and asthenospermia. As a typical antioxidant, MI has a considerable protective effect against DNA oxidative damage and can lessen the effects of oxidative stress on sperm quality and fertility in infertile men. According to Canepa *et al.* (*60*), subfertile male semen quality (sperm concentration, number of spermatozoa, progressive motility, total motile sperm count, and normal sperm morphology) can be improved by a nutraceutical supplement containing MI, folic acid, alpha-lipoic acid, betaine, and vitamins. In a different study, oligoasthenoteratozoospermic individuals who consumed supplements primarily made of MI and added MI to *in vitro* sperm culture demonstrated improved sperm motility both *in vitro* and *in vivo* (*61*). In light of this, MI may enhance sperm function in oligoasthenoteratozoospermic individuals both *in vitro* and *in vivo*. Additionally, Montanino *et al.* (*62*) discovered that MI delivered vaginally could increase pregnancies while also being safe for both the mother and the foetus by boosting total sperm motility and cervical mucus quality.

3.1.5. Coenzyme Q10

Coenzyme Q10 is a crucial coenzyme of the body's oxidative respiratory chain as well as a vital fat-soluble antioxidant. Therefore, by enhancing mitochondrial function, coenzyme Q10 supplementation may enhance pregnancy outcomes in infertile patients. With age, plasma coenzyme Q10 levels decline. In older women, coenzyme Q10 supplementation accelerated oocyte maturation and decreased post-meiotic aneuploidy. Additionally, coenzyme Q10 supplementation can increase the amount of coenzyme Q10 in the follicular fluid of people with infertility. Coenzyme Q10 supplementation boosted clinical pregnancy rates, but there was no discernible difference in miscarriage or live birth rates, according to a systematic review and meta-analysis (*63*).

Coenzyme Q10 has well-known antioxidant properties, and while semen can shield sperm from oxidative stress, treatment with this coenzyme can increase sperm motility (*64*). Coenzyme Q10 is present in sperm cells and spermatoplasm in a predetermined amount, and its degree of concentration is associated with specific sperm characteristics. Therefore, a reduction in coenzyme Q10 levels in sperm may result in a reduction in sperm motility. Improved pregnancy outcomes can be achieved by using coenzyme Q10 to boost sperm mitochondrial energy production and neutralize the ROS generated (*65*). Despite being affordable and secure, coenzyme Q10 is currently not utilized extensively in clinical practice since more patients and improved clinical trial designs are required to completely quantify and confirm its impact on human fertility.

3.1.6. Other antioxidants

When taken in supplement form, vitamin C has been shown to reduce endogenous oxidative damage and enhance sperm quality in infertile men. An important antioxidant is carnitine. To speed up the oxidation process, carnitine functions as a crucial cofactor in the transport of long-chain fatty acids in the mitochondrial matrix. Therefore, carnitine can improve postgonadal maturation, sperm production, and cellular energy generation. According to previous studies, patients with idiopathic asthenospermia who took L-carnitine and acetyl-L-carnitine together experienced significant improvements in their overall motor function (*66*). Resveratrol enhances mitochondrial homeostasis in oocytes and granulosa cells, stimulates mitochondrial biosynthesis, and improves ovarian reserve in older women by regulating the balance between mitochondrial biogenesis and autophagy by activating sirtuin-1 (*67*). Additionally, resveratrol enhances clinical infertility

caused by follicular growth by stimulating nuclear factor-κB and phosphoinositide 3-kinase/protein kinase B. By blocking nuclear factor-κB signaling, the nuclear factor-κB signaling pathway reduces ovarian dysfunction in POI patients and prevents granulosa cell apoptosis (*68*). Its anti-inflammatory qualities, however, might prevent the inflammatory response of decidualization, which would lower endometrial receptivity and have negative consequences on implantation and endometrial decidualization.

3.1.7. Metformin

Caloric restriction (CR), an antioxidant strategy, limits a person's daily calorie intake to between 25% and 50% of their average calorie intake. CR can promote fertility, postpone ovarian senescence, and extend reproductive life. According to Weeg *et al.* (*69*), obese female oocytes have aberrant telomeres, which impact both the amount and quality of oocytes. Telomere shortening and the activation trend of the compensatory mechanism for telomere shortening were more noticeable in the high body mass index group in 20 women who had ART for pregnancy, and telomere length was adversely linked with the number of follicles and fertilization rate. It is difficult to put CR into practice, but there are substitutes that have the same impact. For instance, metformin inhibits mitochondrial ETC complex I and lowers the generation of hepatic glucose, simulating the impact of CR and lowering the production of ROS (*70*).

Meanwhile, the application of metformin attenuates PCOS through the downregulation of miR-122, miR-223 and miR-29a in women (*71*). For patients with recurrent endometriosis, lncRNA H19 is highly expressed in the ectopic endometrium and is an independent prognostic factor of endometriosis (*72*), and the combination therapy of metformin and sitagliptin upregulates lncRNA-H19 by suppressing the PI3K/AKT-DNMT1 pathway in patients with PCOS accompanied by IR (*73*). Thus these ncRNAs can be considered as potential markers in molecular PCOS research and treatment approach development.

3.2. Anticoagulants

Anticoagulants can increase endometrial receptivity during the "window of implantation" and be used to boost implantation rates empirically. Examples include aspirin and low-molecular-weight heparin. Aspirin is a non-selective cyclooxygenase inhibitor that can alter the ratio of prostacyclin to thromboxane, increase blood flow, and enhance endometrial tolerance. The use of low-dose aspirin during *in-vitro* fertilization/intracytoplasmic sperm injection could enhance pregnancy rates, according to the findings of a meta-analysis of 13 randomized controlled studies performed by Li *et al.* (*74*); the clinical dose that was advised was 100 mg/d. In patients with repeated implantation failure, endometrial and uterine artery blood flow resistance was dramatically reduced following low-dose aspirin treatment compared to before treatment (*75*).

By interacting with antithrombin, the sulfated polysaccharide heparin can prevent the activity of clotting proteins such as factor Xa and factor IIa. Insulin-like growth Factor 1 (IGF-1) may control the differentiation and implantation of endometrial tissue. In addition, IGF-1 is upregulated during the early stages of decidualization, which helps the endometrial stromal cells differentiate and is important for endometrial receptivity. Low-molecular-weight heparin can increase gonadal hormone expression while decreasing IGF-1 expression, which can increase the pregnancy rate (*76*). Furthermore, heparin-binding malignant growth factors can encourage angiogenesis and the development of a capillary network at the implantation site of embryos (*77*).

3.3. Levothyroxine

There is currently insufficient evidence for LT4 to increase fertility in females with normal thyroid function and positive thyroid autoimmune antibodies. Hypothyroidism was consistently linked to low folate and high Hcy levels (above 100%). In addition, Lien *et al.* (*78*) showed a correlation between thyroid function, which is elevated in hyperthyroidism and depressed in hypothyroidism, and *MTHFR* gene activity. Therefore, we speculate that adding thyroxine to your diet may boost the activity of the *MTHFR* gene. All women seeking treatment for infertility should be examined for thyroid disease, if found, and treated with low dose LT4, whose initial dose is 25-50 μg/d, due to the safety of this medication (*79*).

3.4. Dehydroepiandrosterone

DHEA is an endogenous steroid that is produced by ovarian follicular membrane cells and the adrenal reticular zone. It serves as a precursor hormone for the production of testosterone and oestradiol. A recent meta-analysis revealed that DHEA improved pregnancy outcomes in patients with decreased ovarian reserve by raising anti-müllerian hormone levels and the number of antral follicles (*80*). Studies have shown that DHEA supplementation increases the number of primordial and primary follicles in aged mice by inhibiting follicular apoptosis (*80*). By upregulating the expression of HOXA-10 in the uterus, Celik *et al.* (*81*) discovered for the first time that oral DHEA supplementation might improve endometrial receptivity in women who were not good responders. DHEA's safety (regular oral dose and usage time) is unclear.

3.5. Glucocorticoid

Due to their ability to control excessive androgen

expression by blocking adrenocorticotropic hormone activation, glucocorticoids have been the primary treatment for CAH. In people with non-classic adrenal hyperplasia, studies have shown that glucocorticoids can increase fertility by lowering excessive levels of testosterone and progesterone (*43*). Ovulation dysfunction in CAH women and supplemention with glucocorticoids may help to induce ovulation. Retrospective studies revealed that glucocorticoids improved fertility by lowering ovulation dysfunction and preventing overexposure of the endometrium to progesterone and androgens from the adrenal glands. Patients with CAH who received glucocorticoids had more regular periods, became pregnant, and had healthy babies (*41*). Additionally, hormone therapy can be used throughout pregnancy with the main goals of restoring cortisol insufficiency, suppressing androgen overproduction, preventing the masculinization of female foetuses and preventing long-term consequences on child fertility (*82*).

Hsa-circ 001533, spliced from FKBP51, is crucial in cell proliferation, migration and apoptosis. It has been reported that FKBP51 interacts with the glucocorticoid receptor (*83*). A recent study has identified that hsa-circ 001533 contributes to the cumulus cell apoptosis and nuclear maturation of oocytes by inhibiting the expression of FKBP51 in infertile women with endometriosis (*84*). Thus, the regulatory effect of glucocorticoids on circRNAs needs further validation.

3.6. Gonadotropin

Due to the oligosaccharide transferase complex of pituitary gonadotropin cell glycosylation of α and β subunits during protein synthesis, FSH is a complex, highly diverse glycoprotein hormone. Hormones control spermatogenesis, therefore, when exogenous gonadotropin or pulsing gonadotropin-releasing hormone is used, testosterone levels in the testis and serum rise and sperm are present during ejaculation. Theoretically, hormones can stimulate and enhance sperm output, which may be the rationale behind FSH treatment of male infertility caused by hypogonadotropic hypogonadism (*85*). Male idiopathic infertility can benefit from gonadotropin treatment by increasing sperm count and function, but not in all cases. More randomized controlled trials are required to prove this, but it may rely on the aetiology of oligoasthenoteratozoospermia and/or the genetic background that affects FSH action. Overproduction of FSH can cause ovarian hyperstimulation syndrome, which can trigger *FSHR* mutations and cause tumours that secrete FSH (*86*). Fortunately, no available research has documented negative effects on men following FSH stimulation.

Granulosa cells in follicles respond to gonadotropin signaling and participate in follicular formation and oocyte maturation. In mice with knockout of miRNA-200b and miRNA-429, the female mice were sterile and regained fertility only after application of exogenous gonadotropins for superovulation (*87*). In anovulatory women, these miRNAs were overexpressed, while exogenous gonadotropins decreased the levels of these miRNAs to a normal level in normal ovulating women (*87*). Therefore, exogenous gonadotropin treatment provides a promising strategy for fertility by inhibiting miRNA-200b and miRNA-429 expression.

4. Future directions

On the basis of the pathogenesis, we outline the current knowledge, including randomized controlled trials and systematic reviews, and suggest potential future approaches for the use of targeted medications on infertility. LncRNA, miRNA, and circRNA are examples of RNA that does not translate proteins and are referred to as ncRNA. They are anticipated to become a novel target for the treatment of reproductive illnesses since they have a significant role in controlling the occurrence and development of reproductive diseases (Figure 3).

4.1. LncRNA

A target gene's mRNA expression is influenced by lncRNA through the competitive binding of miRNA, posttranscriptional regulation, and epigenetic alteration. Yao *et al.* (*88*) discovered 52 differentially expressed messenger RNA transcripts and 20 differentially expressed messenger RNAs that were associated with granulosa cell activity and follicular development. Furthermore, they compared the RNA transcripts of the ovarian cortex and serum between POI patients and normal controls and then found that the expressions of lncRNA-ADAMTS1-1:1/ADAMTS1 and lncRNA-PHLDA3-3:2/CSRP1 in POI patients' ovarian cortex were upregulated significantly and the expressions of lncRNA-COL1A1-5:1/COL1A1, lncRNA-SAMD14-5:3/COL1A1, and lncRNA-GULP1-2:1/COL3A1 were downregulated, according with the results of serological examination. Subsequently, lncRNA-GULP1-2:1 and other potential target genes showed a positive correlation with ovarian tissues, both of which were significantly downregulated. In a rat experiment, Xiong *et al.* (*89*) discovered that lncRNA-MEG3 prematurely caused ovarian failure by inhibiting the proliferation of ovarian granulosa cells and activating the p53-p66Shc pathway and the apoptosis-related protein caspase-3.The synthesis of oestradiol was enhanced when the lncRNA HCG26 was downregulated in granule cells, which also hindered cell growth and cell cycle progression (*90*). In mouse ovarian granule cells, Li *et al.* (*91*) discovered that overexpression of the lncRNA SRA could increase the levels of oestradiol and progesterone as well as the production of vital enzymes (YP19A1 and CYP11A1). The novel lncRNA CASC7 is a potential

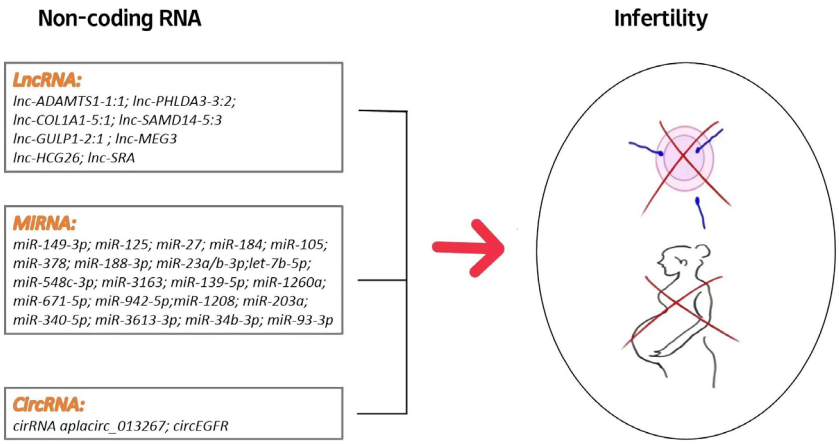

Figure 3. NcRNAs includes lncRNA, miRNA and circRNA, which have a significant role in controlling the occurrence and development of reproductive diseases.

therapeutic target and promising biomarker for male infertility (*92*).

4.2. miRNA

Endogenous miRNAs are short, ncRNAs that affect gene expression at the posttranscriptional or translational level. They are involved in steroidogenesis and germ cell development and contribute to infertility. The miRNA-125 family also plays a crucial regulatory role in oocyte activation. Mature miRNA-125b has two precursors, miRNA-125b-1 and miRNA-125b-2. Li *et al.* (*93*) also found that miRNA-27, miRNA-125b, miRNA-184 and miRNA-105 inhibited the release of progesterone and oestrogen in ovarian granulosa cells and that miRNA-378 could directly regulate oestradiol production.

During various spermatogenic phases, miRNAs are highly expressed. By attaching to the 3' untranslated region of CRISP2 and inhibiting CRISP2 production following transcription, miRNA-27a selectively targets CRISP2, a protein important for sperm motility. A study of oligosthenospermia patients showed that miRNA-23a/b-3p expression was negatively correlated with sperm motility, morphology, and sperm count, and some of the direct targets identified by miRNA-23a/b-3p, including PFKFB4, HMMR, SPATA6, and TEX15, play a fundamental role in sperm function (*94*). Let-7b-5p expression was observed to be lower in asthenozoospermia patients than in healthy males and additional research revealed that low let-7b-5p expression prevented glycolysis by specifically targeting AURKB in people with asthenozoospermia (*95*). In addition, miRNAs have been predicted in infertile males including azoospermia (miRNA-548c-3p), oligozoospermia (miRNA-3163, miRNA-548c-3p, miRNA-139-5p, miRNA-1260a), asthenozoospermia (miRNA-671-5p, miRNA-942-5p, miRNA-1208), and teratozoospermia (miRNA-203a, miRNA-340-5p, miRNA-3613-3p, miRNA-34b-3p, miRNA-93-3p) (*96,97*). Therefore, these miRNAs can be used as novel potential therapeutic targets for patients with infertility.

4.3. CircRNA

CircRNAs are ncRNAs that control posttranscriptional regulation of gene expression. CircRNAs have a crucial role in ovarian growth and function, according to research on circRNAs in the ovary over the past ten years. Comparing granule cells from women with biochemical premature ovarian insufficiency (bPOI) and control women's, Zhou *et al.* (*31*) discovered that in bPOI patients, a total of 133 circRNAs were elevated and 424 circRNAs were downregulated. Further study revealed that the Foxo signaling pathway is one of the signaling pathways that is highly enriched in cellular senescence. Therefore, circRNAs may be involved in the pathogenesis of bPOI. Wu *et al.* (*98*) showed that the circRNA aplacirc_013267 inhibited apla-mi-1-13 and increased the expression of THBS1, upregulating granulosa cell apoptosis. Jia *et al.* (*99*) discovered that *circEGFR* gene knockdown increased progesterone production while inhibiting granulosa cells' ability to secrete oestrogen. Conversely, circEGFR overexpression improved the proliferation and oestrogen production of granulosa cells in mouse ovaries.

5. Conclusion

Adjuvant treatment and drug substitution have been used to treat infertility successfully. To better assist infertile couples in improving the success of pregnancy, it will be necessary to conduct additional research on the effectiveness and safety of these drugs before they can be used as adjuncts for pregnancy. Additionally, the dosage and methods of use of these drugs should also be standardized. Future tailored medications to treat infertility may be guided by the projected potential target genes and signaling pathways discussed above, but more extensive studies are still needed.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Jing Zhou, Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai, China 200011.
E-mail: zhoujing_dan1995@163.com

Ling Wang, Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai, China 200011.
E-mail: Dr.wangling@fudan.edu.cn

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Review

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Advances in understanding the effect and mechanism of dehydroepiandrosterone on diminished ovarian reserve

Jing Wang[1,2,3,§], Xinyao Pan[1,2,3,§], Jing Zhou[1,2,3], Xin Li[4], Yan Sun[1,2,3,*], Ling Wang[1,2,3,*]

[1] Laboratory for Reproductive Immunology, Hospital & Institute of Obstetrics and Gynecology, Shanghai Medical College, Fudan University, Shanghai, China;
[2] The Academy of Integrative Medicine of Fudan University, Shanghai, China;
[3] Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, Shanghai, China.
[4] Urumqi hospital of traditional Chinese medicine, Urumqi, Xinjiang, China.

SUMMARY Diminished ovarian reserve (DOR) refers to the decline in fertility caused by the loss of normal ovarian function. DOR is associated with adverse reactions to ovarian stimulation during *in vitro* fertilization and embryo transfer (IVF-ET), increasing cycle cancellation rates and reducing pregnancy rates. Although it is well known that dehydroepiandrosterone (DHEA) can be used as a dietary supplement for age-related diseases, its potential has gradually been shown for many diseases. In this review, we focus on the effects of DHEA on DOR, briefly analysing its clinical benefits and limitations and describing the mechanism of function and the clinical trials conducted. Therefore, we summarize the mechanisms and indications of DHEA for DOR.

Keywords dehydroepiandrosterone, diminished ovarian reserve, testosterone, *in vitro* fertilization and embryo transfer

1. Introduction

Decreased ovarian reserve (DOR) refers to the loss of the normal reproductive capacity of the ovary due to a decrease in the numbers and quality of remaining eggs. DOR is present in 10-30% of infertility patients, which is a challenge (*1*). Natural ageing leads to a decrease in ovarian reserve, but genetic defects, aggressive medical treatment, certain surgical procedures and injuries may also cause DOR (*2*). DOR is associated with abnormal reproductive results, such as increased embryonic aneuploidy, fertilization failure, and increased miscarriage rates (*3*). Dehydroepiandrosterone (DHEA), which is the most abundant circulating steroid hormone in humans, plays a critical role in various physiological functions (*4*). Oocytes remain in a static state in the unrecruited primordial follicle and do not truly age. Once recruited, they enter an age-related ovarian environment, the quality of which affects the meiotic segregation processes as women age, resulting in aneuploidy. This result suggests that ovarian ageing may be related to the ovarian environment and not limited to the oocyte itself. DHEA may play a role in altering this ovarian environment, thereby preventing follicle ageing (*5*). The role of DHEA in DOR, mainly in artificial reproduction treatment (ART) and *in vitro* fertilization (IVF), is to improve oocyte quality and quantity and significantly improve anti-Müllerian hormone (AMH) levels (*6*). This review provides a critical assessment of the evidence on the use of DHEA supplementation to improve ovarian function in women with DOR.

2. How can DOR be assessed?

DOR is characterized as a decrease in the quantity or quality of the available oocyte pool, an intermediate situation between normal reproductive biology and premature ovarian failure. A decreasing quantity and quality of oocytes in women, usually around the age of 40 years, is a normal physiological phenomenon (*7*). However, some women experience a much earlier than normal decline in ovarian reserve and premature infertility. The main clinical characteristics of DOR include regular period cycles with abnormal ovarian reserve test results but not at postmenopausal levels (*8*). To define DOR, the antral follicular count (AFC) and AMH and follicle-stimulating hormone (FSH) serum levels are the most estimated and frequently used criteria (*9*).

DOR is defined in the Federal Register Notice based on clinically assessed reduced fertility associated with reduced ovarian function and is usually expressed as

an FSH level > 10 mIU/mL or an AMH level < 1.0 ng/mL (*10*). Research on the relationship between ovarian reserve and follicle quality showed that the FSH levels in DOR patients aged < 35 years were ≥ 10 mIU/mL, and the possibility of obtaining immature oocytes in these patients was 4.4-fold higher than that in normal patients. That is, for each unit increase in basal FSH levels, patients were 23% more likely to have an immature oocyte (*11*). In women under 40 years of age, a single abnormal FSH value may not be predictive of DOR, and repeat testing should be performed promptly. When defining DOR patients according to an AMH level < 1.2 ng/mL, women with DOR have a high number of immature and abnormal oocytes, despite having normal FSH levels, suggesting that FSH itself may not be a good indicator of oocyte quality (*12*).

AMH can be considered a major marker of fertility when the ovarian reserve and the pool of growing follicles change (*13,14*). AMH provides a potent marker of the quality of oocytes in young women undergoing controlled ovarian stimulation (COS), as cytoplasmic changes are thought to be the most significant factor in embryo quality and implantation (*15*). Normal perivitelline oocytes were suggested to be associated with a higher fertilization rate and improved embryo development. Some studies were shown that infertile women diagnosed with DOR due to low levels of AMH may also have more follicles in the ovarian cortex than their AMH levels reflect (*16*). Therefore, a low AMH level may not necessarily mean a low number of oocytes (*17*). The linear regression analysis showed that AMH was highly correlated with the cumulative live birth rate in each age group of women, especially with DOR. The inclusion of AMH as a reference indicator will provide more information and personalized prediction of the cumulative live birth rate prior to ART (*18*). However, more supporting research needs to be provided to determine whether AMH can accurately predict follicle numbers.

The AFC refers to the sum of the number of follicles in the two ovaries on Days 2-4 of the menstrual cycle. During *in vitro* fertilization embryo transfer (IVF-ET) treatment, a low AFC predicts a poor ovarian response with high specificity but low sensitivity (*19*). The most commonly reported threshold for predicting adverse reactions is an AFC between 5 and 7 (*20*). AMH levels and the AFC are significant indicators for early identification of DOR before it reaches critical levels (*21,22*). However, the limitations of the AFC lie in the level and judgement of the ultrasound doctor and the tendency of the AFC to overvalue FSH-sensitive follicles and oocyte counts since it inevitably must measure atretic follicles of the same size (*23*).

3. The differences among DOR, POR, and POI

One of the confusing aspects of this topic is that there are several diagnoses and terms associated with DOR. The similarities and differences among concepts related to primary ovarian insufficiency (POI), premature ovarian failure (POF), and poor ovarian response (POR) will be discussed. In clinical diagnosis, POF/POI differs from DOR. DOR is diagnosed by testing for abnormal ovarian reserves in women who have regular menstrual cycles but are not postmenopausal. In contrast, patients diagnosed with POI/POF have postmenopausal FSH levels and no periods for 4 months (*24*). DOR, as a normal physiological process, occurs around the age of 40 years but is pathological if it occurs at a young age. POR refers to a poor ovarian response to IVF stimulation. There is a remarkable overlap between DOR and POR in terms of diagnosis and the corresponding indicators of ovarian reserve. Each specific diagnosis can be found in Table 1.

4. How is DHEA produced and metabolized?

DHEA is a naturally occurring adrenal steroid in mammals. It is synthesized and metabolized from cholesterol to androstenedione and oestrogen (*4*). CYP11A1, a common precursor of all other steroid hormones, transports free cholesterol (FC) to the outer mitochondrial membrane (OMM) through the transport of cytochrome P450 side chain cleavage enzymes (P450scc, CYP11A1) (*25*). Consequent progesterone metabolism produces various bioactive steroids, such as adrenal cortex hormones, ovarian Theca cells, and testicular stromal cells, in a tissue-specific manner (*26*). The synthesis of DHEA is regulated by the hormonal signaling cascade in the hypothalamic-pituitary-adrenal system. Hypothalamus-released corticotropin-releasing hormone stimulates anterior pituitary synthesis and the secretion of adrenocorticotropic hormone (ACTH), partially regulating adrenocortical secretion in adults. Through the ACTH/cAMP/PKA signaling pathway, PKA signals rapidly increase the expression of genes such as STAR, CYP11A1 and CYP17A1 in mitochondria and participate in androgen synthesis and the phosphorylation

Table 1. The diagnostic differences among DOR, POR and POI

DOR	POI	POR	POF
(1).FSH level >10 but nonmenopausal levels (2).AMH level < 1.2 and AFC <10 (3).Failed clomiphene citrate challenge test (4).Regular periods (*10*)	(1).Age < 40 years (2).FSH level > 25 (at least 2 times with an interval of more than 4 weeks) (*63*)	(1).Age ≥ 40 years (2).POR history (3).AMH level < 0.5-1.1 or AFC between 5 and 7 (*64*)	(1).Age < 40 years (2).FSH level > 40 (3).Amenorrhea ≥ 4 months (*65*)

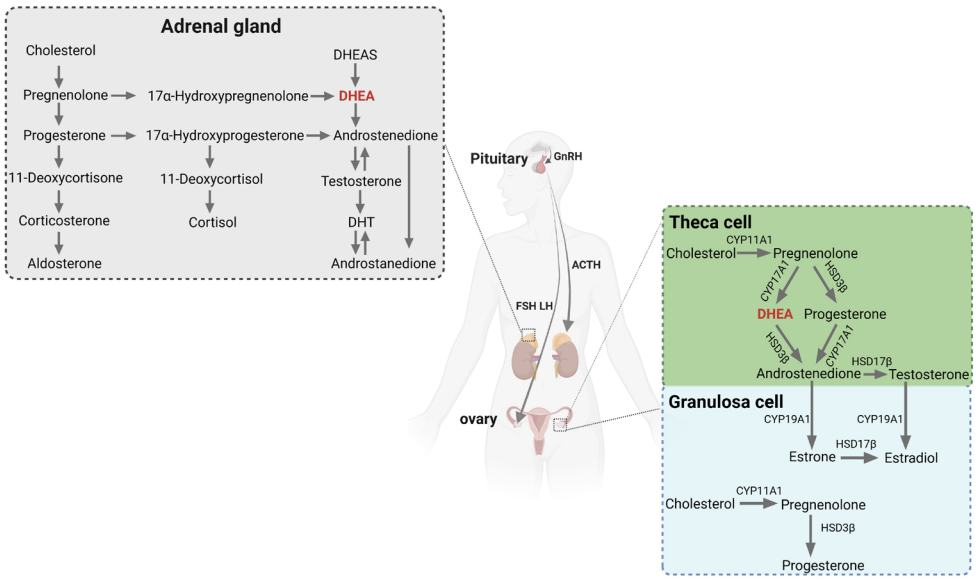

Figure 1. The synthesis and metabolism of DHEA in the adrenal glands and ovaries. The biosynthesis of DHEA involves the secretion of different hormones by the hypothalamus acting on the adrenal glands and ovaries respectively, including ovarian Theca cells, granulosa cells and adrenal cortical cells. Created with BioRender.com.

of transcription factors (*27*). CYP17A1 is an integral membrane protein in the endoplasmic reticulum that transfers electrons from NADPH to CYP17A1 (*28*). DHEA of sulfuric acid can be generated in the adrenal cortex through the 3β′-hydroxyl group. DHEA can be converted to androstenediol by 17β-hydroxyl steroid dehydrogenase (HSD17B) in the adrenal cortex, both of which can eventually be converted to testosterone. Testosterone can transform dihydrotestosterone (DHT) by 5α reductase (SRD5A). The two-cell system cooperates in a specific cycle pattern to stimulate the production of androgens in ovarian Theca cells and oestrogen in granulosa cells (*29*). Testosterone is converted into oestradiol 17β in granulosa cells through the action of CYP19A1 in the theca cells of the ovary (*30*). In short, both the adrenal gland and ovarian tissue can produce T, Δ4A, and DHEA and release them into surrounding tissues for use (Figure 1).

5. Mechanism of DHEA for DOR

The most exclusive function of DHEA is that it can be metabolized to potent androgens and oestrogens (*31*). Supplementation with DHEA appears to produce more precise results and cause fewer adverse side effects than supplementation with testosterone (*32,33*). DHEA converts intracellularly to androgens with no significant active testosterone released into the blood (*33,34*). In addition, DHEA enhances the ovarian response to the androgen pathway and may serve as a ligand for androgen receptor (AR) along with FSH to promote follicle formation through increased numbers of primary, preantral and antral follicles (*35*). With DHEA supplementation, the expression of FSH receptor (FSHR) is enhanced in granulosa cells (*36*) (Figure 2). Studies have also demonstrated that DHEA upregulates serum AMH and inhibin B (INHB) levels as well as paracrine insulin-like growth factor-1 (IGF-1), which contributes to the recruitment of follicles to improve the ovarian response (*37*). However, some studies have reported that AMH or INHB levels did not increase significantly, while a noticeable increase in AFC was observable. Thus, an increased AFC suggests that preventing atresia of the small antral follicles may be one of the potential mechanisms by which DHEA exerts its effect. Zhang *et al.* (*38*) published a meta-analysis of changes in AMH levels and the AFC after DHEA supplementation, which showed that DHEA treatments increased the AFC. In another self-control study, the results showed a significant improvement in AMH levels in the DHEA self-control group (*39*). However, when comparing AMH levels between the DHEA and placebo groups, there were no significant differences observed between pre- and post-supplementation. Therefore, the impact of DHEA supplementation on ovarian reserve markers remains controversial. Further trials are required to investigate the potential effects of DHEA on ovarian reserve markers and pregnancy hormones. DHEA regulates many genes involved in molecular mitochondrial morphological changes. DHEA significantly increases the gene expression of the outer mitochondrial membrane protein mitofusin-1 (MFN1) and regulates optic atrophy 1 (Opa1) fusion of the inner mitochondrial membrane. Although DHEA had difficulty improving the expression of fusion genes, the expression of the split genes DNM1L and mitochondrial fission factor (Mff) was significantly reduced. This also shows that DHEA supplementation greatly reduces the expression of fission genes (*40*). DHEA can increase the function and activity of mitochondria, reduce the apoptosis and

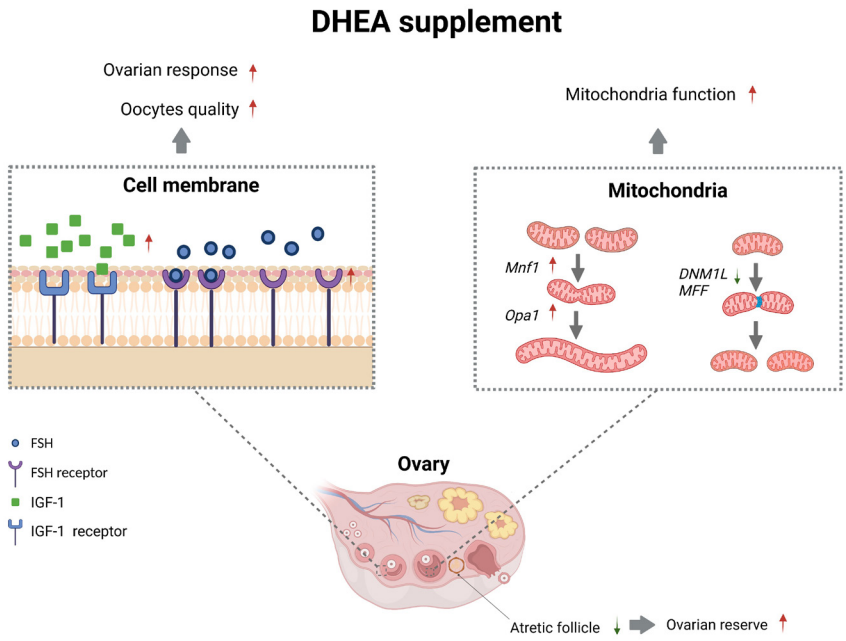

Figure 2. The mechanisms of DHEA for DOR. DHEA increases the expression of FSHR through the AR signal pathway, promotes the growth of preantral follicles, and reduces follicular atresia. DHEA reduces the expression of fission genes DNMIL and MFF genes and enhances the mitochondrial function. Elevated IGF-1 expression can promote follicle maturation and steroid production. Created with BioRender.com.

cell necrosis of cumulus cells (CCs) and human granular cell lines, and delay the ageing of CCs. In addition, DHEA also reduces mitochondrial division and increases the clearance of poorly mitotic mitochondria (*41*). Dr. Narkwichean (*42*) showed that DHEA may slightly alter the microenvironment of the primate ovary by reducing damage to DNA and apoptosis and increasing the mass of mitochondria, the activity of mitochondrial dehydrogenase and the expression of mitochondrial transcription Factor A (TFAM) in CCs (*43*).

6. ART results of DHEA supplementation for DOR

Women with DOR usually respond poorly to COS in ART cycles, and approximately 5-18% of IVF cycles conclude with POR, yielding successful pregnancy rates as low as 2% to 4%. There may be a poor response to COS, which means that fewer oocytes can be obtained according to the standard IVF protocol and that not enough good-quality embryos can be transferred, which leads to a poor reproductive prognosis. Shiqiao Hu (*44*) compared the birth rates of non-DOR patients and DOR patients undergoing ART fresh embryo transfer (ET) cycles. Dr. Yeung (*45*) randomly assigned 32 women to receive DHEA supplementation before IVF treatment. The results showed that pretreatment with DHEA in women undergoing IVF for DOR did not improve their prognosis, while no differences were found in the number of oocytes retrieved, IVF cycle cancellation rates or miscarriage rates (*29,45-47*). However, some retrospective studies revealed the opposite conclusion. Dr. Sonmezer reported that DHEA-treated DOR patients had not only increased numbers of > 17 mm follicles and MII oocytes but also high-quality 2-3 day embryos (*48*). Another report also showed an increased number of > 15 mm follicles, MII oocytes and embryos after DHEA supplementation (*49*). Dr. Kotb MM (*50*) started a study with 140 women with POR who were undergoing ART. Regarding the oocyte count and fertilization rate, the DHEA group had significantly higher clinical pregnancy rates and sustained pregnancy rates. Dr. Yeung's experiments showed a similarity in the size and numbers of oocytes in the two groups, while the fertility rates and numbers of high-quality embryos were higher in the DHEA group (*51*). In a meta-analysis, the clinical pregnancy rates were significantly increased in patients with DOR who received DHEA pretreatment; however, when the data were restricted to randomized controlled trials, there was no statistically insignificant difference in the clinical pregnancy rates (*52*). Regarding the abortion rate, there was no difference between women who received pretreatment with DHEA and those who did not (*46*). A meta-analysis by Xu L (*53*), including 9 prospective randomized controlled trials (RCTs) and 833 patients, showed that patients treated with DHEA had improved oocyte retrieval, clinical pregnancy and live birth rates compared to controls. During the 12 weeks before IVF, subjects received three doses of 25 mg DHEA or placebo daily (*54*).

The POSEIDON classification, which combines ovarian reserve markers with age, COS and other risk factors targeted to treatment, has positive results in predicting adverse effects. Some studies have demonstrated improved oocyte and embryo production and cumulative pregnancy rates in women classified in POSEIDON Group 4 who receive DHEA supplements.

Table 2. Summary of clinical studies on IVF in DOR patients with DHEA treatment

First author/Year	Group	Dosage	Conclusion
MD Ozcil, 2020 (*6*)	DHEA (*n* = 34)	50 mg	DHEA improves ovarian reserve and pregnancy rates in women with POR
Qiaofei H, 2017 (*66*)	DHEA (*n* = 53) Control (*n* = 50)	25 mg	DHEA increased the expression of AR in granulosa cells
Agarwal R, 2017 (*39*)	DHEA (*n* = 20) Control (*n* = 20)	25 mg	DHEA increased AMH expression
Zhang H, 2016 (*67*)	DHEA (*n* = 64) DHEA + climen (*n* = 60)	25 mg	DOR with low FSH levels may benefit more from DHEA and climen supplementation
Haydardedeoğlu B, 2015 (*68*)	DHEA (*n* = 44) Control (*n* = 22)	25 mg	DHEA may reduce aneuploidy in the embryos of women with DOR
Gleicher N, 2013 (*69*)	DHEA (*n* = 44) Control (*n*= 213)	75 mg	DHEA significantly improves ovarian reserve, especially in young women
Weissman A, 2011 (*70*)	DHEA (*n* = 15) Control (*n* = 15)	75 mg	DHEA increases circulating progesterone concentrations
Gleicher N, 2009 (*71*)	DHEA (*n* = 73)	25 mg	DHEA significantly reduces the miscarriage rate in DOR patients over 35 years old
Casson PR, 2000 (*72*)	DHEA (*n* = 5)	80 mg	DHEA improves the ovarian response and reduces the gonadotropin dosage

In addition, DHEA supplementation in patients over 40 years of age may improve cumulative pregnancy rates (*55*). A lack of uniform evidence-based methodology and inclusion criteria exists for clinical studies, as well as heterogeneity in IVF treatment protocols, so there is conclusive evidence on clinical data (Table 2).

7. The dosage, duration and side effects of DHEA supplementation

A meta-analysis included patients with DOR who were treated with DHEA at a dose of 75 mg per day in RCTs (*53*). Another study also showed that most people tolerated a DHEA dose of 75 mg/day well (*56*). The dose of DHEA varies from 50 to 90 mg/day, and the treatment duration ranges from 1 to 12 months. For patients with adrenal insufficiency, the initial dosage of DHEA is 25-50 mg per day orally, with symptomatic improvement and an expected treatment duration of at least 4-6 months. Because DHEA may have side effects similar to those of androgens, lower doses (25-30 mg/day) may be better for long-term treatment (*57*). However, no pharmacological studies have been conducted to determine the optimal dose, duration, or timing of DHEA supplementation in patients with DOR (*58*). As dietary supplements, DHEA formulations are not regulated by the Food and Drug Administration (FDA) for pharmaceutical quality. Therefore, there is no guarantee of standardization of the formulations used (*59*).

Dr. Karp (*60*) demonstrated that DHEA could cause seizures. Furthermore, DHEA may also cause oily skin, acne and unnecessary female- or male-pattern hair growth (hirsutism) (*61*). DHEA should not be taken for seizures by women with liver dysfunction, hypertension, acute manic symptoms, convulsions and palpitations (*60,62*). Further studies are needed to provide more evidence of side effects and to optimize DHEA supplementation for the best efficacy.

8. Conclusion

DHEA is an important steroid in a variety of physiological processes and its therapeutic effects are gradually being shown in research. The exact underlying mechanism by which DHEA affects the ovaries and embryos has not been fully demonstrated, so further evidence of the effects of DHEA pretreatment in patients with DOR and its role in IVF-ET needs to be verified. The differences in the results of different studies may lie in differences in the definition of DOR, the use of COS, the dosage of DHEA and the duration of DHEA treatment. The role of DHEA on DOR needs to be supported by more data.

Further DHEA research has shown DHEA to have great potential for dealing with multiple physiological processes and sheds light on the treatment of several gynaecological diseases. Supplementation with DHEA in women with DOR who are planning to undergo IVF/ICSI remains controversial, and more experiments are needed to prove its effectiveness. A variety of trials including women selected by Bologna criteria have analysed serum levels as well as clinical pregnancy rates as major outcomes to evaluate the effect of DHEA. DHEA achieves its action in DOR patients by multiple mechanisms, which may result in the complex evaluation of the outcomes. However, some of the trials have proven that DHEA does exert an influence on the results, and some have hardly reached statistical significance. Through various randomized and double-blind prospective clinical trials, a standard evaluation system should be built to measure the outcomes more scientifically.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Ling Wang, Obstetrics & Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai, China 200011.
E-mail: dr.wangling@fudan.edu.cn

Yan Sun, Hospital & Institute of Obstetrics and Gynecology, Fudan University, 128 Shenyang Road, Yangpu, Shanghai, China 200081.
E-mail: ysunsh@126.com

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Women undergoing *in vitro* fertilization/intracytoplasmic sperm injection-embryo transfer (IVF/ICSI-ET) might benefit from maintaining serum luteinizing hormone levels: A retrospective analysis

Yingping Xu[1,§], Jia Chen[2,3,§], Yanlin Zhang[2,3], Qing Qi[4,5,6], Jing Zhou[4,5,6], Qi Zhou[7], Danyi Tang[7], Ling Wang[4,5,6,*]

[1] Reproductive Medicine Centre, Taizhou Hospital of Zhejiang Province, Linhai, Zhejiang, China;
[2] College of Acupuncture and Orthopedics, Hubei University of Chinese Medicine, Wuhan, Hubei, China;
[3] Hubei Provincial Collaborative Innovation Center of Preventive Treatment by Acupuncture & Moxibustion, Wuhan, Hubei, China;
[4] Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China;
[5] The Academy of Integrative Medicine of Fudan University, Shanghai, China;
[6] Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, Shanghai, China;
[7] Department of Gynecology, Traditional Chinese Medicine Hospital of Yangpu District, Shanghai, China.

SUMMARY We aimed to evaluate the effect of serum luteinizing hormone (LH) levels on human chorionic gonadotropin (HCG) injection day (LH_{HCG}) on outcomes of *in vitro* fertilization/intracytoplasmic sperm injection-embryo transfer (IVF/ICSI-ET) patients. It is a retrospective cohort study involving 620 women who had an IVF cycle in Taizhou Hospital Affiliated to Wenzhou Medical University between 2018-2020. The participants were divided into different groups according to LH_{HCG} level and age. The clinical data and outcomes were compared between groups. The numbers of follicles (≥ 14 mm) on HCG day, retrieved oocytes, mature oocytes, and two pronuclei (2PN) embryos in women with LH_{HCG} < 2 IU/L were more than those with LH_{HCG} ≥ 2 IU/L. Women with LH_{HCG} < 2 IU/L had lower high-quality embryo rate (42.2% *vs.* 46.5%, $p = 0.002$) and implantation rate (40.0% *vs.* 58.8%, $p = 0.044$) compared to those with LH_{HCG} ≥ 2 IU/L. When LH_{HCG} < 2 IU/L, there was no significant difference in implantation rates in patients < 35 years compared to those ≥ 35 years. When LH_{HCG} ≥ 2 IU/L, patients < 35 years old had higher implantation rates (71.7% *vs*. 41.2%, $p < 0.001$) compared to those ≥ 35 years old. The success rates of IVF fertilization and ICSI fertilization and biochemical and clinical pregnancy rates were not significantly different between groups. Our results demonstrated that women undergoing IVF/ICSI-ET might benefit from maintaining LH_{HCG} levels at ≥ 2 IU/L. In addition, age might associate with LH_{HCG} levels and be a better determining factor of the transfer outcome than serum LH_{HCG} levels for IVF/ICSI-ET.

Keywords luteinizing hormone, human chorionic gonadotropin, IVF/ICSI-ET, gonadotropin-releasing hormone antagonist, pregnancy outcomes

1. Introduction

The quality of follicles is different according to various controlled ovarian hyperstimulation (COH) treatments during *in vitro* fertilization/intracytoplasmic sperm injection-embryo transfer (IVF/ICSI-ET), impacting pregnancy outcomes (*1*). Gonadotropin-releasing hormone (GnRH) agonist (GnRH-a) and GnRH antagonist (GnRH-ant) protocol are clinically used to control the quality of follicles. The GnRH-ant can block the GnRH receptor without causing a premature surge in luteinizing hormone (LH), resulting in a shorter period of ovulation induction, less reliance on gonadotropins (Gn), and fewer cases of severe ovarian hyperstimulation syndrome than the GnRH-a long protocol (*2,3*). However, the main drawback of GnRH-ant administration was a rapid and significant reduction of serum LH levels, which adversely affected oocyte retrieval and embryo quality, resulting in poor outcomes in IVF/ICSI-ET (*4*). Appropriate GnRH-ant protocols have become increasingly prevalent in assisted reproductive technology (ART).

LH, a pituitary hormone regulated by GnRH, interacts with its receptors and promotes the maturation of ovarian follicles. A surge of LH triggers ovulation in the middle of the menstrual cycle, which prompts the corpus luteum to produce progesterone, essential for maturing the uterine endometrium to prepare for the implantation of a fertilized egg (*5*). Low endogenous levels of LH adversely affect the development of normal follicles and the endometrium after ovulation (*6*). A study has shown that low serum LH levels the day following a GnRH-a trigger are linked to a decreased rate of ongoing pregnancies, high miscarriages, and low live births (*7*). In women receiving human chorionic gonadotropin (HCG) with less than 30% LH in the serum, the pregnancy and implantation rate was significantly reduced (*7,8*). However, other contrary findings indicated pregnancy outcomes were similar for patients with low and high serum LH levels (*9-11*). Still, it is unclear whether GnRH-ant should be used based on serum LH levels during COH. Despite the fact that supplementing LH during GnRH-ant administration compensates for the severe decrease in levels of endogenous LH caused by the GnRH-ant (*4*), there is controversy whether patients with endogenous LH deficiency can benefit from GnRH-ant treatment.

In addition, age is supposed to be an independent factor affecting serum LH levels and pregnancy outcomes. Clinical studies suggest that LH-supplemented ovarian stimulation was not beneficial to younger women but that women over 35 are likely to benefit from LH combined with follicle-stimulating hormone (FSH) (*12,13*). Whether exogenous LH supplementation and age factor can effectively improve pregnancy outcomes is an unmet clinical question.

The research was conducted to evaluate the impact of LH level on HCG day (LH_{HCG}) and age on outcomes of IVF/ICSI-ET patients using the GnRH-ant protocol. We retrospectively analyzed 620 women who had undergone IVF/ICSI-ET and accepted the GnRH-ant protocol. Exogenous LH was applied if needed during the GnRH-ant protocol. Fertilization and pregnancy outcomes were investigated from the different groups, which were divided according to LH_{HCG} level (2 IU/L) and age (35 years).

2. Patients and Methods

2.1. Patients and study design

This retrospective study was performed in Taizhou Hospital, Zhejiang Province, from January 2018 to December 2020. Inclusion criteria were as follows: *i*) Aged 20-50 years old; *ii*) underwent IVF/ICSI-ET and accepted GnRH-ant protocol. Exclusion criteria included: *i*) Incomplete study data; *ii*) uterine pathology or morphological abnormality; *iii*) those with gonadal disorders or conditions that affected the secretion or excretion of sex hormones and *v*) chromosome abnormality. Ethical approval was obtained by the Ethics Committee of Taizhou Hospital, Zhejiang Province. During the study, 1276 women were screened based on inclusion and exclusion criteria. Stratified analyses were performed with the patient population according to a level of serum LH (LH_{HCG} < 2 IU/L *versus* $LH_{HCG} \geq 2$ IU/L) and age (< 35 years *versus* ≥ 35 years). In total, given the confounding factors of frozen embryo implantation, only 125 women with fresh embryo implantation were eligible for implantation analysis (Figure 1).

2.2. GnRH-ant protocol

Gn was intramuscularly injected to induce ovulation if serum basal levels of FSH and LH conformed to standard on the 2nd-4th day of menstruation, and no functional follicles were showed by transvaginal ultrasound. The initial gonadotropin dose of Gn (Lishenbao, 75 U, Lizhu Group) varied from 150 to 225 IU according to serum hormone levels and antral follicle count (AFC). During COH, the dose for GnRH-antagonist (Sizekai, 0.25 mg, Merck Cerano, France) injection depended on the follicle's diameter (≥ 14 mm) and serum basal LH levels (> 10 IU/L). Follicular development was monitored on vaginal ultrasound examination and hormone levels. When serum LH level is too low and follicular development is not synchronized, human menopausal gonadotropin (HMG, Lebaode, 75 IU, Lizhu Group, Zhuhai, China) is injected to supplement exogenous LH. When ≥ 3 follicles reached a diameter of ≥ 17 mm, or ≥ 2 follicles reached a diameter of ≥ 18 mm, 5000-10000 IU of HCG was administered. Aspiration of the oocytes was carried out 36 hours after injection of HCG with ultrasound guidance.

2.3. IVF/ICSI-ET

IVF was performed as a routine, while ICSI was conducted in the following cases: *i*) Patients with previous conventional fertilization failure or fertilization rate < 30%; *ii*) spouses with severe oligospermia, asthenospermia, and teratozoospermia; *iii*) obstructive, non-obstructive azoospermia; *iv*) difficult to take sperm or unable to ejaculate after taking eggs; *vi*) cryopreservation of a limited number of sperm. Normal fertilization could be confirmed when two pronuclei (2PN) were visible 16-18 h after insemination. Three days after oocyte retrieval, the decision to perform a transplant was made in accordance with embryo grading, hormone levels, and the condition of the endometrium. A suitable number of embryos was transferred according to Chinese legislation: For the first time in their reproductive years, women under 35 can only be transferred two embryos, whereas women

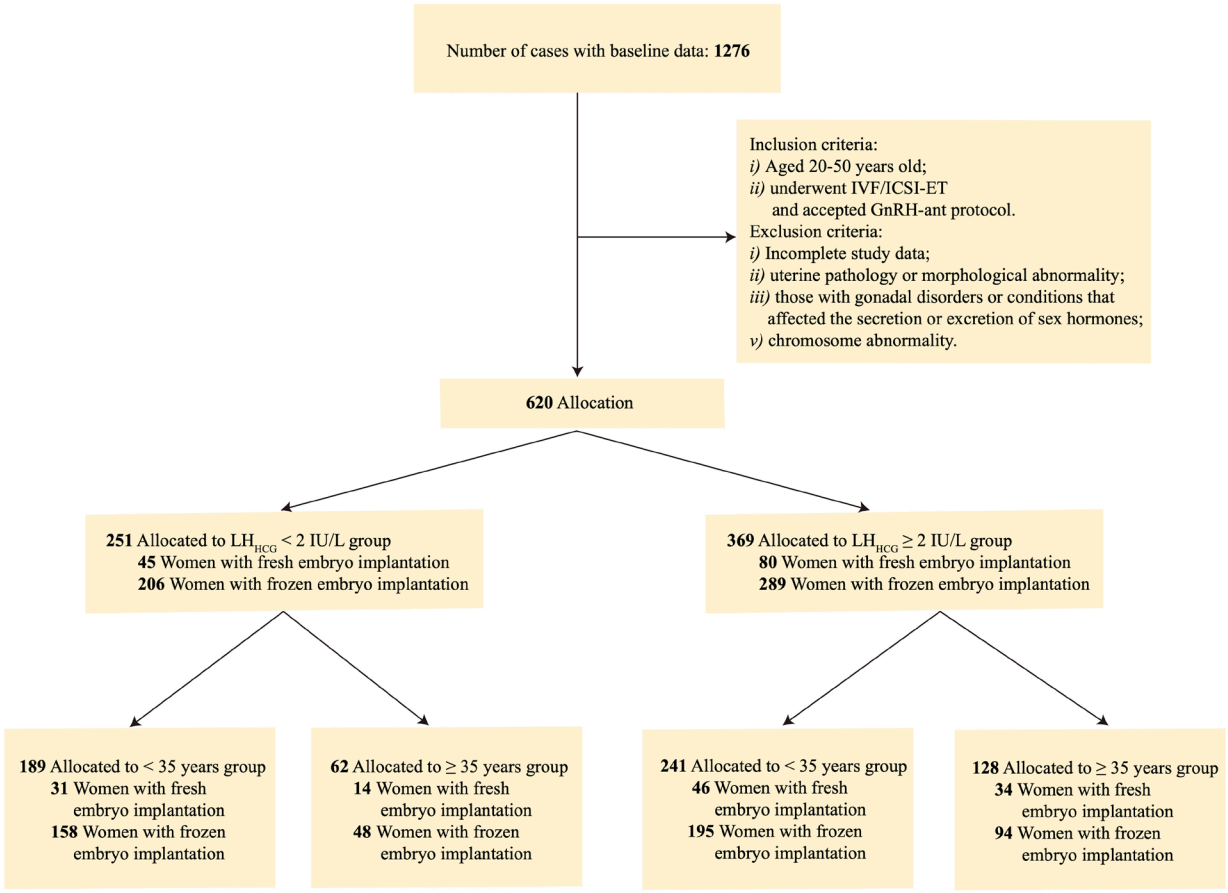

Figure 1. Flow chart of patient disposition

over 35 or who have failed IVF/ICSI-ET several times can receive two to three embryos (*14*). The high-quality blastocysts were freshly transplanted or cryopreserved, and the non-high-quality blastocysts were discarded.

2.4. Outcomes

There were two main outcomes: biochemical and clinical pregnancy rates. Biochemical pregnancy was confirmed as serum β-HCG > 25 IU/L within 12-14 days of embryo transfer (*15*). Clinical pregnancy was confirmed according to gynecological ultrasonography 30-35 days after embryo transfer (*16*). Furthermore, we evaluated the implantation rate, high-quality embryo rate, normal fertilization rate of IVF, and normal fertilization rate of ICSI. Age, body mass index (BMI), infertility duration, bilateral AFC, basal FSH level, basal LH level, basal FSH/LH, basal estradiol, LH_{HCG}, progesterone level on HCG day (P_{HCG}), amount of follicles ≥ 14 mm on HCG day, retrieved oocyte number, mature oocyte number, 2PN embryo count, and high-quality embryo count were also analyzed.

2.5. Statistical methods

In normal distributions, the mean and standard deviation are used to represent continuous variables. In view of the non-normal distribution of the data, continuous variables have been expressed as median (quartile range). When analyzing continuous variables, the Mann-Whitney *U* test was used. With categorical variables expressed as percentages, the Chi-squared test or Fisher's exact probability were used to test the data. Statistical significance was reached at $p < 0.05$, and statistical analyses were conducted with SPSS 25.0 (IBM, Chicago, IL, USA).

3. Results

3.1. Participant characteristics

All 620 patients were divided into LH_{HCG} < 2 IU/L group and LH_{HCG} ≥ 2 IU/L group based on serum LH_{HCG}. There were no differences in BMI ($p = 0.076$) and infertility duration ($p = 0.451$) between the two groups. However, a younger age ($p = 0.006$) was observed in the LH_{HCG} < 2 IU/L group. No significant differences were found in infertility duration, bilateral AFC, basal estradiol, and P_{HCG} between the two groups. Patients in the LH_{HCG} < 2 IU/L group had lower basal FSH levels (7.4 *vs.* 7.8, $p = 0.006$), lower basal LH levels (4.0 *vs.* 4.7, $p < 0.001$), and greater basal FSH/LH (1.83 *vs.* 1.73, $p = 0.049$) (Table 1). Thus, age might associate with LH_{HCG} levels.

3.2. Patients in LH_{HCG} < 2 IU/L group trended to be fertilized but had a lower implantation rate

A suitable range of LH levels for achieving pregnancy has not been established. Some studies conclude LH_{HCG} may predict the fate of IVF, in which LH = 2 IU/L is an important demarcation point (*17,18*). In this study, we investigated the outcomes of fertilization and implantation according to the level of LH. The embryo transfer rate was no significant difference between LH_{HCG} < 2 IU/L group (45/251, 17.9%) and LH_{HCG} ≥ 2 IU/L group (80/369, 21.7%). A comparison of LH_{HCG} groups was carried out to examine fertility and pregnancy outcomes. Compared to LH_{HCG} ≥ 2 IU/L group, patients in the LH_{HCG} < 2 IU/L group had more number of follicles ≥ 14 mm on HCG day, retrieved oocytes, mature oocytes, and 2PN embryos, but lower high-quality embryo rate (42.2% *vs.* 46.5%, $p = 0.002$) and implantation rate (40.0% *vs.* 58.8%, $p = 0.044$). The median number of high-quality embryos (3 *vs.* 2, $p = 0.076$), IVF fertilization rate (75.9% *vs.* 75.5%, $p = 0.710$), ICSI fertilization rate (84.0% *vs.* 83.8%, $p = 0.874$), biochemical pregnancy (17.8% *vs.* 15.0%, $p = 0.684$) rate and clinical pregnancy rate (31.1% *vs.* 43.8%, $p = 0.165$) were no significant differences between two groups (Table 2). Therefore, patients with LH_{HCG} < 2 IU/L intended to be fertilized, while patients with LH_{HCG} ≥ 2 IU/L had a higher implantation rate.

3.3. Subgroup analysis

3.3.1. Participant characteristics in different subgroups

A woman's LH bioactivity typically falls across the threshold of 35 years (*19*). LH_{HCG} < 2 IU/L group and LH_{HCG} ≥ 2 IU/L group were further classified according to age. When LH_{HCG} < 2 IU/L, no difference was observed regarding BMI, infertility duration, basal FSH levels, basal estradiol, and P_{HCG} in different ages; patients in the < 35 years group had more bilateral AFC (15.0 *vs.* 8.5, $p < 0.001$), higher basal LH levels (4.10 *vs.* 3.70, $p = 0.009$), and lower basal FSH/LH (1.76 *vs.* 2.18, $p = 0.006$) than those ≥ 35 years (Table 3). When LH_{HCG} ≥ 2 IU/L, the groups did not differ significantly in BMI and infertility duration in the < 35 years group and ≥ 35 years group. Meanwhile, patients in the < 35 years group had more bilateral AFC (14 *vs.* 9, $p < 0.001$), lower basal FSH levels and FSH/LH ratio, increased basal LH levels (5.20 *vs.* 4.20, $p < 0.001$), higher basal estradiol levels (41.00 *vs.* 36.50, $p = 0.031$), and increased P_{HCG} levels (1.01 *vs.* 0.84, $p < 0.001$) in the LH_{HCG} ≥ 2 IU/L group (Table 4).

Table 1. Characteristics and ovarian reserve of patients with different LH_{HCG} levels

Variables	LH_{HCG} < 2 IU/L	LH_{HCG} ≥ 2 IU/L	*p*
Age (years)	31 (28-34)	32 (29-36)	0.006
BMI (kg/m^2)	22.0 (20.0-24.3)	22.6 (20.3-25.1)	0.076
Infertility duration (years)	3.0 (2.0-5.0)	2.5 (2.0-5.0)	0.451
Bilateral AFC	13 (9-19)	12 (8-19)	0.072
Basal FSH (IU/L)	7.4 (6.3-8.6)	7.8 (6.5-9.4)	0.006
Basal LH (IU/L)	4.0 (3.0-5.4)	4.7 (3.5-6.4)	< 0.001
Basal FSH/LH	1.83 (1.34-2.53)	1.73 (1.18-2.33)	0.049
Basal estradiol (pg/mL)	37.0 (27.0-54.0)	39.2 (27.0-52.5)	0.678
P_{HCG} (ng/mL)	1.06 (0.73-1.47)	0.97 (0.66-1.40)	0.189

LH: luteinizing hormone; HCG: human chorionic gonadotropin; LH_{HCG}: LH levels on HCG day; BMI: body mass index; AFC: antral follicle counting; FSH: follicle stimulating hormone; P_{HCG}: progesterone levels on HCG day.

Table 2. Embryological and pregnancy outcomes of patients with different LH_{HCG} levels

Variables	LH_{HCG} < 2 IU/L	LH_{HCG} ≥ 2 IU/L	*p*
Embryo transfers (*n*, %)	45/251 (17.9)	80/369 (21.7)	0.264
Number of follicles ≥ 14 mm on HCG day	9.0 (6.0-13.0)	7.0 (4.0-11.5)	< 0.001
Number of oocytes obtained	10.0 (6.0-15.0)	8.0 (5.0-13.0)	< 0.001
Number of mature oocytes	10.0 (6.0-14.0)	7.0 (4.5-12.0)	< 0.001
Number of 2PN embryos	8 (5-12)	6 (3-10)	< 0.001
Normal fertilization rate of IVF (*n*, %)	2237/2949 (75.9)	2678/3549 (75.5)	0.710
Normal fertilization rate of ICSI (*n*, %)	2237/2664 (84.0)	2678/3195 (83.8)	0.874
Number of high-quality embryos	3 (1-5)	2 (1-5)	0.076
High-quality embryo rate (*n*, %)	944/2237 (42.2)	1246/2678 (46.5)	0.002
Implantation rate (*n*, %)	18/45 (40.0)	47/80 (58.8)	0.044
Biochemical pregnancy rate (*n*, %)	8/45 (17.8)	12/80 (15.0)	0.684
Clinical pregnancy rate (*n*, %)	14/45 (31.1)	35/80 (43.8)	0.165

LH: luteinizing hormone; HCG: human chorionic gonadotropin; LH_{HCG}: LH levels on HCG day; 2PN: two pronuclei; IVF: *in vitro* fertilization; ICSI: intracytoplasmic sperm injection.

Table 3. Patients characteristics in the LH_{HCG} < 2 IU/L group

Variables	LH_{HCG} < 2 IU/L		*p*
	< 35 years	≥ 35 years	
Age (years)	30 (27-32)	37 (36-40)	< 0.001
BMI (kg/m^2)	22.03 (19.53-24.37)	21.85 (20.65-24.30)	0.450
Infertility duration (years)	3.0 (2.0-5.0)	3.0 (1.0-6.3)	0.774
Bilateral AFC	15.0 (10.0-20.0)	8.5 (6.0-12.3)	< 0.001
Basal FSH (IU/L)	7.30 (6.25-8.50)	7.50 (6.68-8.90)	0.394
Basal LH (IU/L)	4.10 (3.20-5.50)	3.70 (2.60-4.45)	0.009
Basal FSH/LH	1.76 (1.30-2.31)	2.18 (1.50-2.90)	0.006
Basal estradiol (pg/mL)	36.00 (27.00-53.35)	44.00 (26.75-54.75)	0.464
P_{HCG} (ng/mL)	1.09 (0.77-1.54)	0.95 (0.55-1.36)	0.096

LH: luteinizing hormone; HCG: human chorionic gonadotropin; LH_{HCG}: LH levels on HCG day; BMI: body mass index; AFC: antral follicle counting; FSH: follicle stimulating hormone; P_{HCG}: progesterone levels on HCG day.

Table 4. Patients characteristics in the LH_{HCG} ≥ 2 IU/L group

Variables	LH_{HCG} ≥ 2 IU/L		*p*
	< 35 years	≥ 35 years	
Age (years)	30 (27-32)	37 (36-40)	< 0.001
BMI (kg/m^2)	22.70 (20.00-25.10)	22.50 (20.55-25.00)	0.766
Infertility duration (years)	3 (2-5)	2 (1-6)	0.264
Bilateral AFC	14 (10-20)	9 (6-12)	< 0.001
Basal FSH (IU/L)	7.40 (6.30-8.85)	8.60 (7.23-10.28)	< 0.001
Basal LH (IU/L)	5.20 (3.60-7.15)	4.20 (3.33-5.30)	< 0.001
Basal FSH/LH	1.44 (1.00-2.14)	2.05 (1.65-2.78)	< 0.001
Basal estradiol (pg/mL)	41.00 (29.00-54.00)	36.50 (24.00-50.68)	0.031
P_{HCG} (ng/mL)	1.01 (0.73-1.54)	0.84 (0.53-1.26)	< 0.001

LH: luteinizing hormone; HCG: human chorionic gonadotropin; LH_{HCG}: LH levels on HCG day; BMI: body mass index; AFC: antral follicle counting; FSH: follicle stimulating hormone; P_{HCG}: progesterone levels on HCG day.

3.3.2. When LH_{HCG} ≥ 2 IU/L, patients < 35 years had higher implantation rates than those ≥ 35 years

Better implantation and pregnancy rates have been reported when patients aged ≥ 35 years had LH supplementation added to COH protocol, while others disagree (*20,21*). In this study, subgroup-wise differences analyses were performed to investigate whether adding exogenous LH in women ≥ 35 years benefits under the IVF/ICSI-ET. The results revealed that the number of follicles ≥ 14 mm on HCG day, retrieved oocytes, mature oocytes, and 2PN embryos were significantly higher in the < 35 years group than those in the ≥ 35 years group, regardless of whether LH_{HCG} < 2 IU/L (Table 5) or LH_{HCG} ≥ 2 IU/L (Table 6). Statistics did not show any significant differences in embryo transfer rate, normal fertilization rate on IVF or ICSI, high-quality embryos rate, biochemical pregnancy rate, and clinical pregnancy rate between the < 35 years group and ≥ 35 years group, no matter whether LH_{HCG} < 2 IU/L (Table 5) or LH_{HCG} ≥ 2 IU/L (Table 6). But when LH_{HCG} ≥ 2 IU/L, patients < 35 years old had higher implantation rates (71.7% *vs.* 41.2%, $p = 0.006$) (Table 6). Maintaining serum LH_{HCG} levels in the range of ≥ 2 IU/L may benefit women under the IVF/ICSI-ET and GnRH-ant protocol. In addition, age may be a better determining factor of the transfer outcome than serum LH_{HCG} levels for IVF/ICSI-ET.

4. Discussion

In this study, serum LH_{HCG} levels were analyzed to determine if they affected outcomes in IVF/ICSI-ET. The results revealed that patients with LH_{HCG} ≥ 2 IU/L had better ovarian reserve capacity, a higher rate of high-quality embryos, and a higher implantation rate. Among patients with low and high levels of LH_{HCG}, there were no statistical differences with regard to clinical outcomes, including fertilization rates in IVF, fertilization rates in ICSI, number of high-quality embryos, biochemical pregnancy rates, and clinical pregnancy rates, in line with previous findings (*9-11,22,23*).

In IVF, GnRH-ant protocols are increasingly preferred, compared to GnRH-a long protocols, because they align with physiological processes (*2*). Women undergoing an IVF/ICSI-ET program are routinely given GnRH-ant to prevent premature LH surges (*24*). Through competitively blocking the GnRH receptor, GnRH-ant administration could rapidly suppress pituitary LH secretion (*25*). Regulating follicle growth and ovulation by FSH and LH is

Table 5. Embryological and pregnancy outcomes of patients in the LH_{HCG} < 2 IU/L group

Variables	LH_{HCG} < 2 IU/L		*p*
	< 35 years	≥ 35 years	
Embryo transfers (*n*, %)	31/189 (16.4)	14/62 (22.6)	0.340
Number of follicles ≥ 14 mm on HCG day	11.0 (7.0-14.0)	6.5 (4.8-9.0)	< 0.001
Number of oocytes obtained	11.0 (9.0-16.0)	7.0 (4.0-10.3)	< 0.001
Number of mature oocytes	10 (7-15)	6 (4-9)	< 0.001
Number of 2PN embryos	9 (5-13)	6 (3-8)	< 0.001
Normal fertilization rate of IVF (*n*, %)	1851/2444 (75.7)	386/505 (76.4)	0.738
Normal fertilization rate of ICSI (*n*, %)	1851/2206 (83.9)	386/458 (84.3)	0.843
Number of high-quality embryos	4 (2-6)	2 (1-4)	0.009
High-quality embryo rate (*n*, %)	766/1851 (41.4)	178/386 (46.1)	0.087
Implantation rate (*n*, %)	15/31 (48.4)	3/14 (21.4)	0.087
Biochemical pregnancy rate (*n*, %)	6/31 (19.4)	2/14 (14.3)	0.681
Clinical pregnancy rate (*n*, %)	12/31 (38.7)	2/14 (14.3)	0.101

LH: luteinizing hormone; HCG: human chorionic gonadotropin; LH_{HCG}: LH levels on HCG day; 2PN: two pronuclei; IVF: *in vitro* fertilization; ICSI: intracytoplasmic sperm injection.

Table 6. Embryological and pregnancy outcomes of patients in the LH_{HCG} ≥ 2 IU/L group

Variables	LH_{HCG} ≥ 2 IU/L		*p*
	< 35 years	≥ 35 years	
Embryo transfers (*n*, %)	46/241 (19.1)	34/128 (26.6)	0.111
Number of follicles ≥ 14 mm on HCG day	8 (5-13)	5 (3-7)	< 0.001
Number of oocytes obtained	10 (7-15)	5 (3-8)	< 0.001
Number of mature oocytes	9 (6-14)	5 (3-7)	< 0.001
Number of 2PN embryos	8 (5-12)	4 (2-6)	< 0.001
Normal fertilization rate of IVF (*n*, %)	2089/2769 (75.4)	589/780 (75.5)	0.968
Normal fertilization rate of ICSI (*n*, %)	2089/2481 (84.2)	589/714 (82.5)	0.275
Number of high-quality embryos	3 (2-6)	2 (1-3)	< 0.001
High-quality embryo rate (*n*, %)	965/2089 (46.2)	281/589 (47.7)	0.515
Implantation rate (*n*, %)	33/46 (71.7)	14/34 (41.2)	0.006
Biochemical pregnancy rate (*n*, %)	7/46 (15.2)	5/34 (14.7)	0.949
Clinical pregnancy rate (*n*, %)	24/46 (52.2)	11/34 (32.4)	0.077

LH: luteinizing hormone; HCG: human chorionic gonadotropin; LH_{HCG}: LH levels on HCG day; 2PN: two pronuclei; IVF: *in vitro* fertilization; ICSI: intracytoplasmic sperm injection.

one of the key factors to improve the success rate of ART. Studies have indicated that FSH and LH surges promote extracellular matrix synthesis and expansion in cumulus cells, contributing to oocyte meiosis. This is crucial for oocyte maturation, ovulation, fertilization, and early embryonic development (*26,27*). A serum LH threshold of < 0.5 IU/L has been proposed as a diagnostic criterion for severe luteinizing depression after IVF ovarian stimulation (*28*). Clinical studies have shown that LH levels below 0.5 IU/L in GnRH-ant treatment significantly lower the rate of implantation and pregnancy. For patients with low levels of LH, adding low-dose urinary HCG improves implantation and live birth rates (*29*). An adequate serum LH level of 1.2 IU/L is required to support FSH-induced follicular development (*30,31*). Thus, adjusting LH levels is of great significance for protecting embryo development in ART. However, relevant studies are disputable about whether endogenous LH should be replenished and which is a suitable level of LH_{HCG} (*32*). In this study, results demonstrated that patients with LH_{HCG} ≥ 2 IU/L had higher implantation rates than those with LH_{HCG} < 2 IU/L when patients ≥ 35 years; therefore, maintaining LH_{HCG} levels in the range of ≥ 2 IU/L can be suitable, especially in women ≥ 35 years.

FSH/LH ratio and bilateral AFC reflect ovarian reserve, which can predict ovarian reserve degradation and COH response (*33*). Studies have shown that the ratio of FSH/LH is positively correlated with age (*34*). It is consistent with our subgroup analysis result that women aged < 35 years had lower FSH/LH and higher bilateral AFC, indicating that patients aged < 35 years had better ovarian reserve capacity. Our study also observed that the basal FSH/LH in LH_{HCG} < 2 IU/L group was higher than that in the LH_{HCG} ≥ 2 IU/L group, which means that patients with LH_{HCG} ≥ 2 IU/L have a better ovarian reserve.

The quality of follicles obtained by hyperstimulation is far more important than the number of follicles. A complex intrafollicular process that controls oocyte maturation affects oocyte quality and impedes embryo implantation (*35*). ARTs have been challenged by the need to select embryos with higher implantation potential (*36*). Our study indicated that high LH levels

are not conducive to follicle excretion and maturation in the early stages of ovulation stimulation, but LH_{HCG} ≥ 2 IU/L is associated with high-quality embryos and implantation. Previous studies have shown that supplementing LH activity in the mid-follicle produces favorable pregnancy outcomes in low-responders (*37*). LH activity administered only in low doses of HCG can support the final stages of follicular development without causing premature luteinization, which supports our theory (*38*).

Increased serum estradiol levels during ovarian hyperstimulation can be good predictive properties for IVF-ET outcomes (*39*). Although there is no direct association between P_{HCG} and clinical pregnancy among women undergoing ovarian stimulation with gonadotropins (*40*), progesterone can cause endometrial thickening, congestion, gland hyperplasia, and branching after ovulation, which is conducive to pregnancy and embryo development. This study showed that estradiol levels remained high during ovarian stimulation, and no significant difference was found between estradiol and P_{HCG} levels according to LH_{HCG} levels. However, when LH_{HCG} ≥ 2 IU/L, patients with age < 35 had higher levels of estradiol and P_{HCG}, suggesting that LH_{HCG} ≥ 2 IU/L and age < 35 can be used as an indication for IVF/ICSI-ET outcomes.

It can be concluded that with age, the quantity and quality of follicles obtained by superovulation stimulation and the quality of embryos gradually decrease, consistent with previous studies (*41,42*). Notably, when patients' LH_{HCG} < 2 IU/L, no difference was observed in implantation rate among different age groups, while when patients' LH_{HCG} ≥ 2 IU/L, patients < 35 years had a higher implantation rate. The result suggests that patients aged ≥ 35 years are more likely to require serum LH_{HCG} levels in the range of ≥ 2 IU/L to meet the needs of embryo transfer and later development.

In conclusion, it is meaningful to monitor and control LH_{HCG} levels to improve ovarian reserve function and blastocyst implantation after transplantation during GnRH-ant protocols because of higher rates of high-quality embryos and implantation. Exogenous LH supplementation may be efficacious in improving pregnancy outcomes in this study; still, the specific level of LH_{HCG} needs further prospective research.

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**Address correspondence to:*
Ling Wang, Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai 200011, China.
E-mail: Dr.wangling@fudan.edu.cn

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Targeting cellular gaps using Janus nanoparticles containing cationic polymers and surfactant lipids

Akihiro Matsumoto[1,*,§], Takeo Kitazawa[2,§], Yuta Hatori[2], Hiroshi Nakanishi[2], Chie Watanabe[3], Tomoya Takashima[1], Masahiro Murakami[1]

[1] Laboratory of Pharmaceutics, Faculty of Pharmacy, Osaka Ohtani University, Osaka, Japan;
[2] Yasuda Women's University, Hiroshima, Japan;
[3] Laboratory of Clinical Pathology, Faculty of Pharmacy, Josai University, Saitama, Japan.

SUMMARY Since nanoparticles are taken up into cells by endocytosis, phagocytosis, or pinocytosis, they have been studied as intracellular drug carriers. Janus particles have an anisotropic structure composed of two or more distinct domains and have been proposed for use in various applications, including use as imaging agents or nanosensors. This study aimed to clarify the influence of the type of nanoparticles on their distribution in a human Caucasian colon adenocarcinoma (Caco-2) cell monolayer. We fabricated Janus and conventional spherical nanoparticles composed of pharmaceutically applicable ingredients. Janus and spherical nanoparticles composed of a cationic polymer and surfactant lipids were prepared by controlling the solvent removal pattern from the oil phase in the solvent removal process using the solvent evaporation and solvent diffusion methods. The distribution of nanoparticles in the Caco-2 cell monolayer was then evaluated using confocal laser microscopy. The mean hydrodynamic size of the fabricated Janus nanoparticles was 119.2 ± 4.6 nm. Distribution analysis using Caco-2 cells suggested that Janus nanoparticles were localized around the adherens junctions located just below the tight junction. Clear localization was not observed in non-Janus nanoparticles with the same composition. The clear localization of the Janus nanoparticles around the adherens junction may be due to their positive charge and asymmetric structure. Our results suggest the considerable potential for the development of nanoparticulate drug carriers to target cellular gaps.

Keywords human Caucasian colon adenocarcinoma (Caco-2) cell, adherens junction, cationic polymer, surfactant lipids, cellular uptake

1. Introduction

Recently, nanoparticles have been used for medical purposes as nanomedicines. They have been investigated as drug carriers for intracellular delivery because they can be taken up into target cells by endocytosis, phagocytosis, or pinocytosis. The first step in cellular uptake is the attachment of particles to the cell membrane. This is influenced by the characteristics of the nanoparticles, such as charge and size (*1-7*). In addition to these properties, the structure of the particles influences their attachment to cell membranes (*1,4,6,7*). Moreover, all the forementioned attributes are cardinal for phagocytic internalization (*1,6*).

Janus particles are representative heterogeneous particles, which have an anisotropic structure composed of two or more distinct domains. They have been proposed for use in various applications, including their use as catalysts (*8,9*), imaging agents or nanosensors (*8,10*), and excipients in cosmetics (*8*). They also exhibit various configurations (*11-13*), including dumbbell, hamburger, spherical, and snowmen structures (*14*). Recently, various medical applications of Janus particles have been proposed. One such proposal involves the application of multiple containments within a Janus particle. For example, Janus particles can achieve dual loading and release of drugs (*15*), which conventional monolithic particles cannot. The distinct domains of Janus particles have different properties, such as being hydrophobic/hydrophilic (*16*) and cationic/anionic (*17*). Hence, these particles can encapsulate specific drugs with different properties in distinct domains or independently release each drug after a different trigger, such as heat (*18*) and light (*19*). We reported that both hydrophobic and hydrophilic fluorescent substances could be encapsulated into Janus microspheres (*20*). Janus particles can also

have separate functions in their distinct domains, including drug-loading and targeting domains. Polymer-magnetic Janus particles can have separate functions in three compartments, that is, anti-tumor drug, magnetic, and fluorescence regions for killing cancer cells, target location, and fluorescence tracing, respectively (*21*). In addition to these applications for drug delivery systems, Janus particles have also been investigated for bio-sensing technologies (*22*).

Another development direction for Janus particles is utilizing their orientational properties, which can occur between particles or particles and membranes. These orientational interactions are a unique property of Janus particles and cannot be achieved using conventional monolithic particles. The orientation between particles is referred to as self-assembly, which can build unique structures (*23-25*). Regarding the orientation of attached particles on a membrane, it has been reported that hollow microtubes can be formed by designing two biologically distinguishable regions: one exhibiting high binding affinity for cells and the other being essentially resistant to cell binding (*26*). In our previous study, orientational attachment on the human Caucasian colon adenocarcinoma (Caco-2) cell membrane was confirmed using Janus-type microspheres composed of a cationic polymer and hard fat (*20*). We found that the Janus microspheres had unique orientation properties in that the cationic side was in contact with the surface of the Caco-2 cell membrane while the lipid side faced the gastrointestinal lumen. This suggests that Janus microspheres are mucoadhesive drug carriers that are not affected by digestive enzymes, which is useful for improving enteral drug absorption by increasing drug retention. However, since Janus microspheres are too large to be taken up by Caco-2 cells and remain on the cell membrane surface, they cannot be used as drug carriers for intracellular delivery. Meanwhile, it has been reported that liposomes and polymeric micelles containing nucleic acids such as small interfering RNA (siRNA), which has low cellular uptake due to its anionic and highly hydrophilic properties, were incorporated into cells by endocytosis (*27,28*). Furthermore, these nanomedicines efficiently allowed siRNA to migrate to the cytoplasm while avoiding endosomal-lysosomal degradation (*27,28*). However, the cell surface dynamics and intracellular distribution of Janus nanospheres, which were prepared by reducing the size of Janus microspheres to facilitate cellular uptake, have not yet been elucidated, and their application as drug carriers for intracellular delivery has not been investigated.

In this study, we first fabricated nanoparticles and investigated the distribution of Janus nanoparticles in the Caco-2 monolayer. Aiming for future use as a delivery system for gastrointestinal absorption of drugs, we used pharmaceutically applicable ingredients for the fabrication of Janus nanoparticles, such as ethyl prop-2-enoate, methyl 2-methylprop-2-enoate, and trimethyl-[2-(2-methylprop-2-enoyloxy) ethyl] azanium chloride (Eudragit® RSPO; Eudragit) and 2-[2,3-bis(2-hydroxyethoxy) propoxy] ethanol, hexadecanoic acid, and octadecanoic acid (Labrafil® M2130CS; Labrafil). We also evaluated the influence of particle structure on Caco-2 intracellular distribution of two types of nanoparticles, Janus and non-Janus. These two types of nanoparticles had the same formulation composition and were prepared by controlling the solvent removal pattern of the oil phase during the solvent removal process, using either the solvent evaporation or diffusion methods. The intracellular distribution of these nanoparticles in Caco-2 cells was analyzed with confocal laser microscopy. Our study findings could potentially focus nanomedicine research on the application of Janus nanoparticles in a new direction.

2. Materials and Methods

2.1. Materials

Eudragit was purchased from Evonik Industries AG (Essen, Germany), while Labrafil was gifted by Gattefossé (Lyon, France). Polyvinyl alcohol (PVA; JP03) was obtained from JAPAN VAM & POVAL Co., Ltd. (Osaka, Japan). Nile Red was purchased from Nacalai Tesque (Kyoto, Japan). A water-soluble, 4′,6-diamidino-2-phenylindole (DAPI)-containing compound (DAPI Fluoromount-G®) was purchased from Southern Biotech (Birmingham, AL, USA). Alexa Fluor™ 488-conjugated mouse monoclonal anti-zonula occludens-1 (ZO-1) antibody (ZO1-1A12; 339188) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate-buffered saline (D-PBS) were purchased from FUJIFILM Wako Chemical Corporation, Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Nichirei Biosciences, Inc. (Tokyo, Japan). All other chemicals used were of reagent grade.

2.2. Cell lines and cell culture

Caco-2 cells were acquired from Dr. Nonaka (Yasuda Women's University). The cells were routinely incubated and maintained in DMEM supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Observation of phase separations and viscosity measurement

Mixtures of Eudragit and Labrafil (1:2 w/w) were dissolved in methylene chloride or acetone. The solvent was slowly evaporated at 25°C until phase separation was observed. The resulting solution was then weighed to calculate the concentrations of the mixtures. For phase separation at the micro-scale, the mixture was

dropped on a glass slide, mounted with a cover glass, and observed using an optical microscope (Motic BA210E, Shimazu Rika Co., Ltd., Tokyo, Japan). The viscosities of the phase-separated solutions were measured using a rotational rheometer (LVDV-I Prime; AMETEK Brookfield Inc., Middleboro, MA, USA).

2.4. Fabrication of nanoparticles

Four types of nanoparticles were fabricated by the following method (Figure 1).

(*i*) Janus nanoparticles and sole Eudragit nanoparticles (method A)

Janus nanoparticles were fabricated using an oil-in-water (o/w)-type emulsion-solvent evaporation method. Here, 60 mg of Eudragit and 120 mg of Labrafil (for Janus particles) or 180 mg Eudragit (for sole Eudragit nanoparticles) were dissolved in 0.01% Nile Red-methylene chloride (1.5 mL) to prepare the oil phase. The resulting oil phase was emulsified in 0.001 M HCl-10% PVA-60% propylene glycol (5 mL) at 20,000 rpm using a homogenizer (ULTRA-TURRAX T18; IKA®-Werke GmbH & Co. KG, Staufen im Breisgau, Germany) for 5 min at 20-23°C. The resulting o/w emulsion was added to 100 mL of water, and the diluted emulsion was stirred at 20-23°C for 120 min to remove the solvent. This process was referred to as solvent evaporation. The obtained nanoparticle suspension was then washed, suspended in 0.01 µM HCl, and concentrated *via* ultrafiltration technique using a 100 kDa filter. The Eudragit and Labrafil particles fabricated using this method were encoded as formulation ID 'NJ' (Nano Janus; NJ) because they showed a Janus structure in nano-order. Furthermore, the sole Eudragit particles were encoded as formulation ID 'NC' (Nano Cation; NC) because they showed a cationic property in nano-order.

(*ii*) Eudragit -Labrafil non-Janus nanoparticles (method B)

Non-Janus nanoparticles were fabricated using the solvent-diffusion method. In this method, 60 mg Eudragit and 120 mg Labrafil were dissolved in 0.002% Nile Red-acetone (10 mL) to prepare the oil phase. The resulting oil phase was poured into 0.5% PVA-0.1 M phosphate buffer solution (pH 8.0) 200 mL under constant stirring. The resulting mixture was stirred, and the solvent evaporated under airflow. The obtained nanoparticles were washed, resuspended in 0.1 M phosphate buffer solution (pH 8.0), and concentrated *via* ultrafiltration using a 100 kDa filter. The particles fabricated using this method were encoded as formulation ID 'NM' (Nano Mixture; NM) because Eudragit and Labrafil were mixed to form particles, unlike Janus nanoparticles in which Eudragit and Labrafil were separated and localized.

(*iii*) Sole Labrafil nanoparticles (method C)

In this method, 180 mg Labrafil was dissolved in 0.01% Nile Red-methylene chloride (1.5 mL) and evaporated under airflow. The resulting mixture was melted at 80 °C, added to water at 80°C (15 mL), and sonicated for 1 min at 20 W using an ultrasonic homogenizer (BRANSON Sonifier SFX250, Emerson

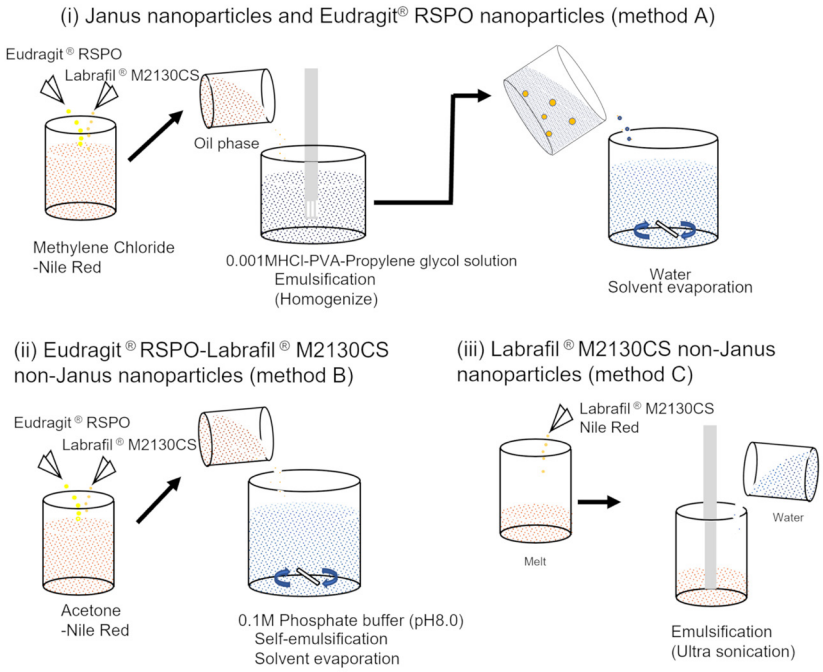

Figure 1. Scheme of the methods used for fabricating the four types of nanoparticles.

Electric Co. Ltd., St. Louis, MO, USA). The particles fabricated using this method were encoded as formulation ID 'NL' (Nano Lipid; NL) because they composed of lipids in nano-order.

2.5. Microscopic observations

The nanoparticles obtained were observed using scanning electron microscopy (SEM) (JSM-5500LV, JEOL Ltd., Tokyo, Japan). Samples for SEM observation were prepared by depositing gold-palladium at 15 mA for 30 s using ion sputtering equipment (Quick Auto Coater JFC-1500, JOEL Ltd., Tokyo, Tokyo, Japan). Samples were observed under the following conditions: accelerating voltage 15 kV, working distance 21 mm, and spot size 15 or 30.

2.6. Characterization of nanoparticles

The size of the nanoparticles was measured by Photon Correlation Spectroscopy using dynamic light scattering (DLS) (ELSZ-2, Otsuka Electronics Co. Ltd., Hirakata, Japan). Water was used as the dispersion medium at 25°C, and the sizes obtained from the DLS analyses were considered the hydrodynamic diameters. Zeta potential was measured using a zeta potential analyzer (ELSZ-2000Z, Otsuka Electronics Co., Ltd., Hirakata, Japan).

2.7. Distribution analysis of nanoparticles in a Caco-2 cell monolayer using immunocytochemistry

The Caco-2 cells were seeded on a porous Transwell filter membrane (cell culture insert, Transparent polyethyleneterephthalate membrane, 24 wells, pore size = 0.4 µm, Corning Inc., New York, USA) at a density of 1×10^4 cells/well and cultured in DMEM containing 10% FBS, 50 U/mL penicillin, and 50 µg/mL streptomycin until a confluent monolayer was formed and its transepithelial electrical resistance (TEER) was approximately 1000 Ω cm^2. Each nanoparticle encapsulating Nile Red was suspended in DMEM supplemented with 10% FBS, 50 U/mL penicillin, and 50 µg/mL streptomycin. After the cells in the apical side of the well were washed with 0.5 mL DMEM with 10% FBS, 50 U/mL penicillin, and 50 µg/mL streptomycin at 37°C, a 0.5 mL aliquot of the resulting suspension with a nanoparticle concentration of 0.4 mg/mL was added to the apical side of each well. After incubation for 3 h at 37°C, the apical side of the well was carefully washed twice with D-PBS. Cells cultured on porous filter membranes were then fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in D-PBS, and blocked with 3% bovine serum albumin. After the porous filter membrane from which cells were cultured was separated from the Transwell using a cutter, the cells were incubated with Alexa Fluor™ 488-conjugated monoclonal anti-ZO-1 antibodies (4°C, overnight). The cells were then mounted using DAPI Fluoromount-G ZO1, a tight junction protein, then the nucleus and nanoparticles in each cell were visualized *via* confocal laser microscopy using a FV1000 microscope (Olympus Co., Ltd., Tokyo, Japan). Images were processed using ImageJ software (*29*) with the Fiji package (*30*).

2.8. Statistical analysis

All experiments were performed in triplicate and the results are presented as mean ± standard deviation.

3. Results

3.1. Phase separation between Eudragit and Labrafil

The phase separation between Eudragit and Labrafil (1:2, w/w) in methylene chloride was observed at 1128 ± 0.3 mg/g at 25°C. Microscopy images of the phase separation and deposition on the surface of the Eudragit -Labrafil (1:2, w/w)-methylene chloride solution is shown in Figure 2. The viscosity of the oil phase of Eudragit -Labrafil (1:2, w/w) at the phase separation point was 54 ± 0.0 mPa·s.

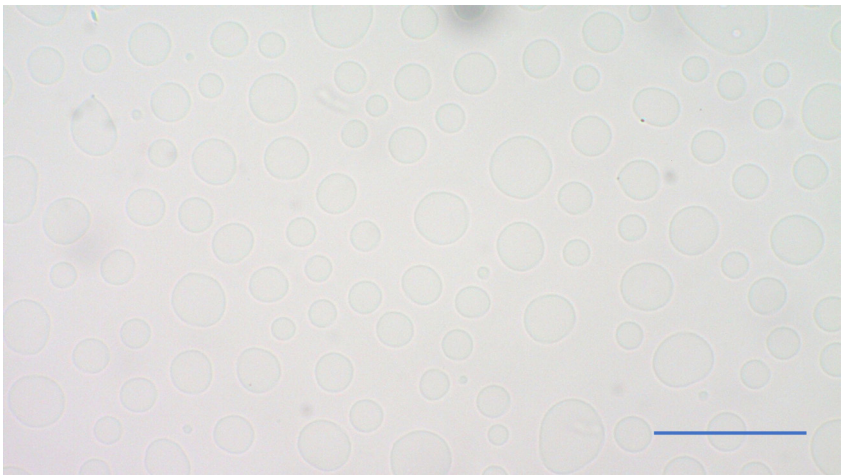

Figure 2. Optical micrograph of the Eudragit® RSPO-Labrafil® M2130CS methylene chloride solution. Phase status after 1 d of drying. The ratio of Eudragit® RSPO/Labrafil® M2130CS was 1:2 (w/w). The scale bar indicates 50 µm.

3.2. Characteristics of nanoparticles

The nanoparticles composed of Eudragit -Labrafil were fabricated using two distinct methods, viz. solvent diffusion and solvent evaporation. The structures of the fabricated nanoparticles are shown in Figure 3 and summarized in Table 1. The Eudragit -Labrafil nanoparticles (NJ01) fabricated using the solvent evaporation method showed a Janus-type structure (Figure 3a), which was independent of Nile Red loading (NJ03) or particle size (NJ02). In contrast, the sole Eudragit (NC01) and sole Labrafil (NL01) nanoparticles had a non-Janus spherical structure. Similarly, Eudragit -Labrafil nanoparticles (NM01) fabricated using the solvent diffusion method showed a non-Janus spherical structure (Figure 3b).

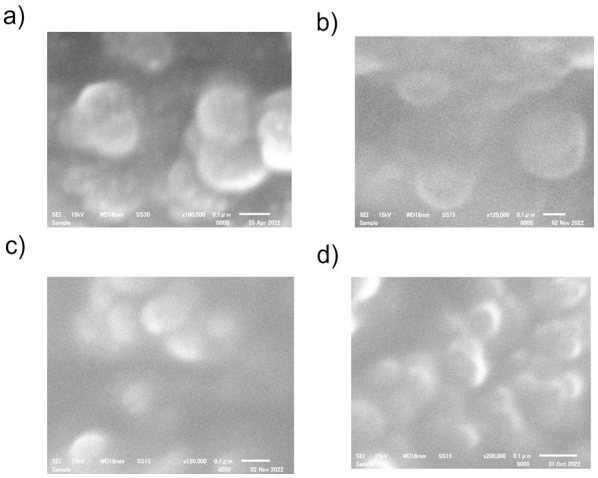

Figure 3. Scanning electron micrograph of nanoparticles. a) Eudragit® RSPO-Labrafil® M2130CS nanoparticles prepared using the solvent evaporation method, method A (NJ01); The ratio of Eudragit® RSPO/Labrafil® M2130CS was 1:2 (w/w). **b)** Eudragit® RSPO-Labrafil® M2130CS nanoparticles prepared using the solvent diffusion method, method B (NM01); The ratio of Eudragit® RSPO/Labrafil® M2130CS was 1:2 (w/w). **c)** Eudragit® RSPO nanoparticles prepared using the solvent evaporation method, method A (NC01). **d)** Labrafil® M2130CS nanoparticles prepared by ultrasonicating the suspension in water, method C (NL01).

The other particle characteristics are summarized in Table 1. Two Janus nanoparticle formulations with different hydrodynamic diameters (the diameter of NJ01 was smaller than that of NJ02) were prepared by adjusting the PVA concentration and stirring speed during emulsification. The measured hydrodynamic diameters of all formulations aligned with the sizes determined *via* SEM, as shown in Figure 3. The hydrodynamic diameters of the spherical Eudragit -Labrafil nanoparticles (NM01) and sole Labrafil nanoparticles (NL01) were larger than those of the smaller Janus (NJ01) and sole Eudragit nanoparticles (NC01); however, their sizes corresponded to those of the larger Janus particles (NJ02).

Table 1 shows the ζ potential of each nanoparticle. The ζ potentials of the sole Eudragit nanoparticles (NC01) and sole Labrafil nanoparticles (NL01) were positive and negative, respectively. Janus nanoparticles (NJ02) were approximately 300 nm in size and were comparable in charge to sole Eudragit nanoparticles (NC01). Small Janus nanoparticles (NJ01) showed a lower ζ potential than larger Janus nanoparticles (NJ02), with a persistently stronger charge than that of non-Janus nanoparticles (NM01).

3.3. Distribution of nanoparticles in Caco-2 cell monolayers

The following five nanoparticle formulations encapsulating the fluorescent dye Nile Red were added to the confluent Caco-2 cell monolayers: Janus nanoparticles (NJ01 and NJ02; the diameter of NJ01 was smaller than that of NJ02) and non-Janus nanoparticles (NM01, NC01, NL01). The distribution of these nanoparticles after 3 h of incubation with the Caco-2 cell monolayers was analyzed *via* confocal laser scanning

Table 1. Characteristics of the fabricated nanoparticles

Formulation No.	Structure	Composition	Nile Red	Fabrication method	ζ potential (mV)		Hydrodynamic diameters (nm)		Polydispersity index	
					mean	S.D.	mean	S.D.	mean	S.D.
NJ01	Janus	Eudragit RSPO: Labrafil M2130CS =1:2	+	Method A	+29.0	8.2	119.2	4.6	0.183	0.057
NJ02	Janus	Eudragit RSPO: Labrafil M2130CS =1:2	+	Method A*)	+41.9	1.8	310.7	5.6	0.183	0.001
NJ03	Janus	Eudragit RSPO: Labrafil M2130CS =1:2	-	Method A	+26.4	5.4	140.1	4.3	0.211	0.072
NM01	Non-Janus (spherical)	Eudragit RSPO: Labrafil M2130CS =1:2	+	Method B	+11.7	1.6	300.4	25.9	0.232	0.029
NC01	Non-Janus (spherical)	Eudragit RSPO	+	Method A	+38.0	5.0	165.8	33.1	0.208	0.029
NL01	Non-Janus (spherical)	Labrafil M2130CS	+	Method C	-32.0	8.8	263.0	34.4	0.243	0.024

Data represents mean ± S.D. (n = 3 batches); *Emulsification condition was adjusted; water phase 2.5% PVA-60% propylene glycol, emulsification speed 2000 rpm using a propeller mixer.

microscopy (Figure 4). Janus nanoparticle formulations were localized both inside and outside the cells near the adherens junction, which were closer to the basement membrane than the tight junction. In contrast, non-Janus nanoparticles (NM01), which consisted of Eudragit and Labrafil, were more widely distributed in the cytosol than Janus nanoparticle formulations (NJ01, 02) and strongly aggregated in cells despite having the same composition.

Herein, we investigated whether the localization of the two Janus nanoparticle formulations (NJ01, 02), near the adherens junction, was due to their strong positive ionic charge or Janus structure. Spherical nanoparticle formulations, consisting only of the cationic polymer Eudragit (NC01) and only Labrafil (NL01), were prepared and added to a Caco-2 cell monolayer, and their intracellular distributions were analyzed. Nanoparticle formulation NC01 (ζ potential: +38.0 mV), containing nanoparticles with a large ζ potential composed only of the cationic polymer Eudragit, also tended to be localized near the adherens junction on the basal membrane side of the tight junction. Moreover, no aggregation was observed, showing a similar tendency to that of nanoparticle formulations NJ01 and NJ02. However, they were distributed in the cytoplasm at the height of the Z-position where ZO-1, a tight junction marker, differed from Janus particles. In contrast, nanoparticle formulation NL01 (ζ = -32.0 mV), which was composed solely of the lipid Labrafil and had a negative ζ potential, was distributed within cells. Moreover, this formulation was observed to have strong particle aggregation and was not distributed outside the cells near the adherens junction, similar to that of the spherical nanoparticle formulation NM01. In addition, because nanoparticle formulation NL01 consisted only of lipids with a low softening point, it was assumed that it strongly aggregated in the cytoplasm after being taken up by cells.

4. Discussion

To investigate the distribution of nanoparticles in cells, their physicochemical properties, including their structure, must be controlled. The structure of the particles is considered to be influenced by the speed of solvent removal from the oil phase into the water phase. In the solvent diffusion process, solvent removal was rapid that Labrafil and Eudragit simultaneously solidified and formed spherical nanospheres. In contrast, the solvent evaporation process takes more time to remove the solvent from the oil phase in the emulsion. In this method, phase separation between Eudragit and Labrafil has to be considered during solvent evaporation. We previously reported that the shape of the resultant particles depends on solvent removal speed (*31*). The relationship between Janus particle formation, phase

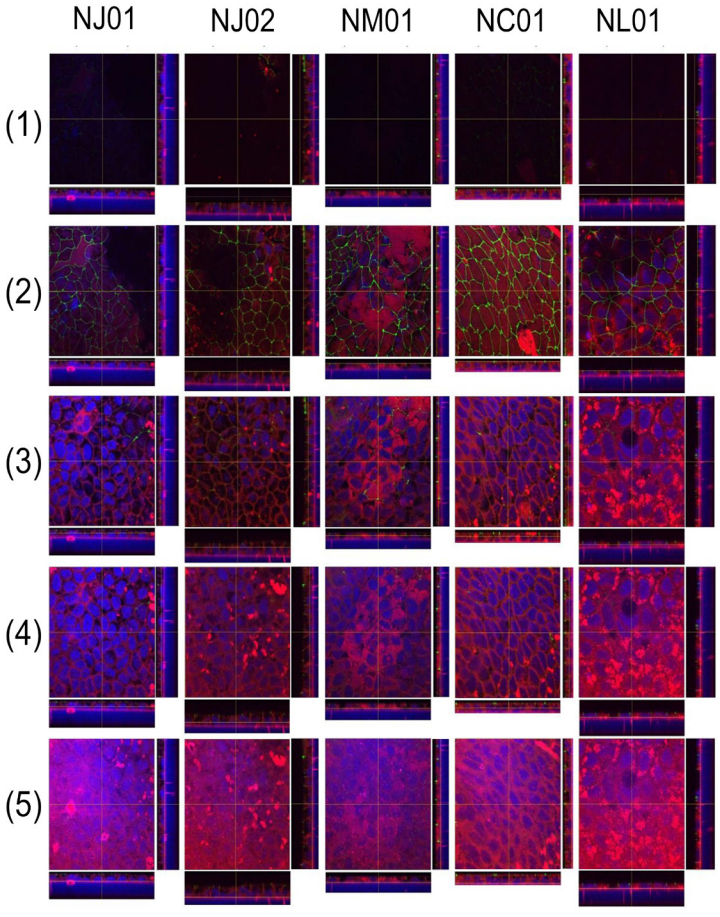

Figure 4. Distribution of nanoparticle formulations within Caco-2 cell monolayers. Five nanoparticles were used, including small Janus nanoparticles of Eudragit® RSPO-Labrafil® M2130CS (NJ01; the ratio of Eudragit® RSPO/Labrafil® M2130CS was 1:2 [w/w]), large Janus nanoparticles of Eudragit® RSPO-Labrafil® M2130CS (NJ02), non-Janus particles of Eudragit® RSPO-Labrafil® M2130CS (NM01; the ratio of Eudragit® RSPO/Labrafil® M2130CS was 1:2 [w/w]), Eudragit® RSPO nanoparticles (NC01), and Labrafil® M2130CS nanoparticles (NL01). Confocal laser microscopy images of Caco-2 cells after 3 h of incubation with each particle (0.2 mg/well). The confocal micrographs represent a three-dimensional analysis of five different optical sections (Z-axis) of the cells as follows: (1) apical membrane, (2) tight junction, (3) adherens junction (closer to the apical membrane), (4) adherens junction (closer to the basal membrane), and (5) basal membrane. The red dots, green lines, and blue spheres indicate nanoparticles stained by Nile Red, tight junctions, and nuclei, respectively.

separation, solvent removal speed, and the viscosity of the oil phase is illustrated in Figure 5.

In the solvent evaporation method, phase separation is supposed to occur in the oil phase at a certain time during the solvent evaporation process. To form a Janus structure, the separated droplets must migrate to the surface of the oil phase in the emulsion during solvent evaporation. The viscosity of the oil phase is important for migration of the separated droplets. The viscosity of the oil phase at the phase separation point between the two materials should not be high enough to separate easily into two layers. In addition, the Janus structure must be maintained after migration. Therefore, the appropriate solvent removal speed is important for maintaining the Janus structure. In this study, the appropriate solvent removal rate was obtained from the volume of the water phase to that of methylene chloride. To this end, we fixed 100 mL as the volume of the water phase in the solvent evaporation process, which provided approximately 1.5 times the saturated solubility (1.3 g/100 mL at 20°C) for the methylene chloride applied in our previous study (*20*).

The structure of particles consisting of two components (1 and 2) is also considered to be influenced by interfacial tension (*32,33*). The role of interfacial tension in nanoparticle structure is illustrated in Figure 6. If phase separation occurs between components 1 and 2 in the oil phase during an o/w type emulsion solvent evaporation, a triple junction comprising components 1- and 2-rich phases in an oil droplet and a water phase is assumed to form. Three interfacial tensions at the triple junction are also suggested to be a determinant of Janus particle formation. Because the Labrafil used in this study had hydrophilic surfactant action (HLB 9), it was assumed to have a sufficiently low surface tension to be in contact with the water phase. Similarly, Eudragit has an increased cationic charge and swells under acidic conditions. In this study, a slightly acidic water phase was used to reduce the interfacial tension between the water and the Eudragit phases for Janus particle formation because Eudragit retains considerable water from swelling. In addition, acidic conditions promoted swelling and protrusion of the Eudragit phase from the Labrafil layer, which precipitated out first. These factors promoted the formation of Janus nanoparticles. In contrast, in solvent diffusion, which produced Eudragit -Labrafil nanoparticles with non-Janus structures, the alkalinity (pH 8.0) of the external phase used in the fabrication process suppressed the swelling of Eudragit with a positive charge. This increased the interfacial tension between the water and Eudragit phases, and the protrusion of the Labrafil layer was suppressed.

Besides the structure of nanoparticles, charge is another important property because it can influence cell membrane attachment and cellular uptake. In this study, Eudragit and Labrafil, used as components of nanoparticles, had positive and negative charges, respectively. However, the Janus and non-Janus nanoparticles consisting of the two components had positive charges. The charge of Eudragit as a cationic polymer was due to its ionic property, whereas that of Labrafil, a non-ionic substance, was due to its dipole property. Generally, the dipole force is weaker than the

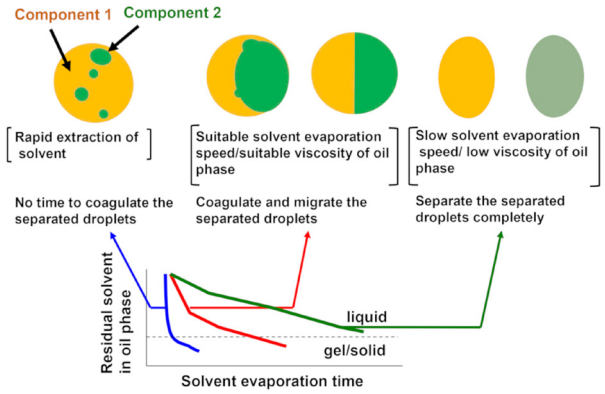

Figure 5. Theoretical image of the influence of solvent evaporation speed on particle structure.

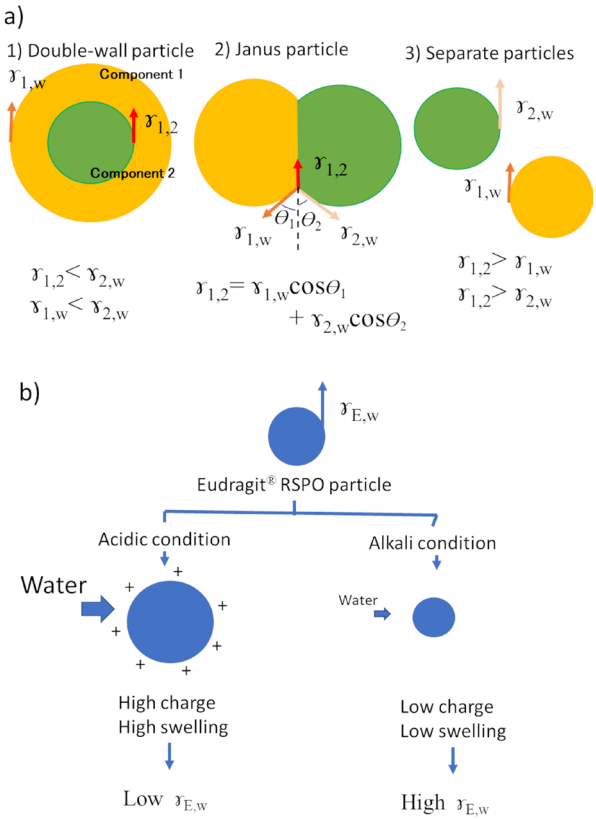

Figure 6. Theoretical image for the role of interfacial tension and swelling on particle structure. a) Effect of interface tension balance on particle structure. The arrows indicate the interfacial tension between components 1 and 2 (red: $\gamma_{1,2}$), between component 1 and external water phase (orange: $\gamma_{1,w}$), and between component 2 and external water phase (peach: $\gamma_{2,w}$). **b)** Effect of acidity and alkalinity in the external water phase on the interfacial tension of cationic polymers. The blue arrow indicates the interfacial tension between Eudragit® RSPO and the external water phase ($\gamma_{E,w}$).

ionic force; therefore, a positive charge dominated on the spherical surface of the Janus particles composed of Eudragit and Labrafil. The non-Janus particles comprising Eudragit and Labrafil showed weaker ζ potentials than the sole Eudragit nanoparticles, indicating that Labrafil shields the positive charge of Eudragit. Complete shielding, similar to the coating of Labrafil over Eudragit, was not likely to occur because the charge of the spherical nanoparticles did not align with the charge of the sole Labrafil nanoparticles. In contrast, in the case of Janus nanoparticles, the positive charge of Eudragit was not shielded by Labrafil similar to non-Janus nanoparticles; therefore, the charge was stronger than that of non-Janus nanoparticles, and the intensity of the charge was comparable to that of sole Eudragit nanoparticles. This was due to the limited shielding at the joint between the two hemispheres.

Next, we investigated the distribution of Janus and non-Janus particles with the aforementioned properties in the intestinal membrane. Janus and non-Janus particles were added to Caco-2 cell monolayers. When the nanoparticles were added to the apical side of the Caco-2 monolayer at 4 mg/mL, as previously reported (*20*), fragmentation of the cell nuclei was observed. Therefore, subsequent nanoparticle incubation experiments were performed at a concentration of 0.4 mg/mL. Nuclear fragmentation was not observed at this concentration. Regarding the cytotoxicity of lipid nanoparticles, the cell viability after 24 h was approximately 70-100% after adding the nanoparticles at 0.4 mg/mL to Vero and L1210 cells (*34*). However, because the lipids and cells used in lipid nanoparticles and their incubation time with cells in this study differed from those in the previous report, further research is required to accurately determine the cytotoxicity of the Janus and non-Janus nanoparticles we used. As a result, unlike the Janus microparticles reported previously (average particle size: 30 μm) (*20*), the Janus nanoparticles of all formulations were taken up by Caco-2 cells. One of the reasons why all nanoparticles were incorporated into the cells could be because of their small particle size, which is known to influence cellular uptake (*6,7*). It has been reported that poly(DL-lactide-co-glycolide) particles (100 nm) were observed inside Caco-2 cells, whereas poly(DL-lactide-co-glycolide) particles larger than 300 nm were only associated with the apical membrane of Caco-2 cells (*35*). However, the results of our experiments suggest that nanoparticles with a diameter of up to 300 nm were taken up into cells, but further research is needed to determine the size threshold for cellular uptake of the carriers.

The main results presented here suggest that the unique intracellular distribution of Janus nanoparticle formulations, which were localized near the adherens junction, was in part due to a strong positive charge. For the non-Janus nanoparticles, the incorporated spherical nanoparticles were distributed throughout the cytosol, which is similar to findings in a previous report (*35*). The nanoparticles of the cationic polymer (NC01) were distributed in the cytoplasm at the height of the tight junction. However, they tended to be distributed near the adherens junction, in contrast to Janus particles. According to these observations, the distribution of Janus particles near the adherens junction may have been due to its positive charge. Meanwhile, the distribution of Janus particles (NJ01, 02) in the cytoplasm at the height of the tight junction was suppressed, which was unique in Janus nanoparticles. Although this may have been due to the Janus structure, further investigation is needed.

Additionally, in this study, there was a difference in the aggregation of Janus and non-Janus nanoparticles in the cell membrane. Non-Janus nanoparticles (NM01) exhibited intracellular aggregation, which was attributed to their low ζ potential (+11.7 mV) due to the shielding of the cationic polymer by the surfactant lipids as mentioned above. Janus nanoparticles had higher ζ potentials. These results suggest that these particles were highly cationic, effectively preventing aggregation.

Unlike conventional lipid nanoparticles, which tend to go to endosomes and lysosomes after cellular uptake by endocytosis or phagocytosis, one way to utilize the unique intracellular distribution of our reported Janus nanoparticles in the cellular gap near the adherens junction can be to use them as drug carriers for the treatment of disorders such as Alzheimer's and inflammatory bowel diseases. Recently, it has been reported that proteases "gingipains" secreted by periodontal disease bacteria "*Porphyromonas gingivalis*" may destroy the blood-brain barrier and cause the progression of Alzheimer's disease (*36*). Furthermore, in inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis with intestinal hemorrhage, free heme released from red blood cells impairs the barrier function of gastrointestinal epithelial cells, further worsening the condition (*37,38*). As a fundamental treatment for such diseases in which barrier disruption of endothelial or epithelial cells is involved in development and progression, we suggest a new method to repair barrier functions by delivering drugs to cellular gaps using Janus nanoparticles containing cationic polymers and surfactant lipids.

In future studies, we will investigate how the Janus nanoparticle formulations, which have cationic surfaces of their Janus structure, interact with cellular gap-related proteins, such as E-cadherin and nectin, to elucidate their detailed intracellular distribution mechanism. E-cadherin and nectin are transmembrane proteins that are bound to actin filaments, forming the cytoskeleton in the cytoplasm near the adherens junction (*39*).

In conclusion, Janus particles were fabricated using the cationic polymer, Eudragit, and lipid, Labrafil. The hydrodynamic size of the fabricated Janus nanoparticles was approximately 100 nm. Distribution analysis of the nanoparticles in Caco-2 cell membrane suggested that the Janus nanoparticles were localized in the adherens

junction and the cytosol near the adherens junction, which is just below the tight junction. Localization was not observed in spherical nanoparticles with the same composition. The clear localization of the Janus nanoparticles around the adherens junction may be due to their positive charge and asymmetric structure. The ability of our Janus nanoparticles to target cellular gaps can lead to their clinical application in the treatment of disorders related to cellular gaps, such as Alzheimer's disease and inflammatory bowel diseases. Although further investigation is needed to elucidate the localization of EudragitLabrafil Janus nanoparticles and suggest possible clinical applications, the present results present considerable potential for the development of nanoparticulate drug carriers to target cellular gaps.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Akihiro Matsumoto, Laboratory of Pharmaceutics, Faculty of Pharmacy, Osaka Ohtani University, 3-11-1 Nishikori-kita, Tondabayashi-shi, Osaka 584-0854, Japan.
E-mail: matumoaki@osaka-ohtani.ac.jp

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Original Article

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Antioxidant, antiglycation, and anti-inflammatory activities of *Caesalpinia mimosoides*

Soraya Rodwattanagul[1], Wutigri Nimlamool[2,3], Siriporn Okonogi[1,3,*]

[1] Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand;
[2] Department of Pharmacology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand;
[3] Research Center of Pharmaceutical Nanotechnology, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand.

SUMMARY Oxidative stress, glycation and inflammation are the main causes of many severe diseases. To date, no single extract has been shown to simultaneously inhibit these three reactions. In this study, the antioxidant, antiglycation and anti-inflammatory activities of ethanol extracts from four edible plants that are commonly used as Thai folk medicine were compared. Among these extracts, *Caesalpinia mimosoides* extract (CME) showed the highest antioxidant potential with Trolox equivalent antioxidant activity (TEAC) of 5.9 ± 0.1 mM/mg followed closely by *Zingiber officinale* extract (ZOE) with a TEAC value of 5.4 ± 0.2 mM/mg. However, CME showed no cytotoxicity, whereas ZOE greater than 60 μg/mL showed cytotoxicity to normal human cells. Antiglycation assay using bovine serum albumin-ribose showed comparable potency between CME and *Spondias dulcis* extract (SDE). However, CME exhibited a high anti-inflammatory activity, significantly higher than SDE and activity depending on the dose. At a concentration of 60 μg/mL, approximately 85% of the interleukin-6 pro-inflammatory cytokine produced from human monocytes, induced by lipopolysaccharides, was completely inhibited by CME whereas SDE showed no inhibition. In summary, CME is the most potential extract with simultaneously activity of these three reactions. CME has the highest total phenolic content expressed as gallic acid equivalent to 301 ± 8 mg/g. Identification using high-performance liquid chromatography revealed the presence of at least four phenolic compounds, gallic acid, syringic acid, *p*-coumaric acid, and ellagic acid are existed in CME. Our finding suggests that CME is a promising natural source for inhibition of oxidative stress, glycation, and inflammation.

Keywords Antioxidant, antiglycation, anti-inflammation, plant extract, chemical composition

1. Introduction

Oxidative stress, glycation, and inflammation are the major causes of several severe chronic non-communicable diseases including diabetes, cardiovascular disorders, and cancers that have become the leading cause of death and morbidity processes (*1–3*). It is known that oxidative stress is a transversal phenomenon in aerobic systems. It occurs when there is an imbalance between the generation of reactive species and inadequate antioxidant defense systems. It has been reported that oxidative stress implicated with the etiology of those severe diseases (*4*). Glycation is a non-enzymatic reaction between the carbonyl group of reducing sugars and the amine group of proteins or nucleic acids to form fructosamine products called Amadori (*5*). The obtained Amadori products continue a series of reactions to form the stable advanced glycation end-products (AGEs) (*6*). These AGEs cause alteration of the structure and function of extracellular matrix proteins (*7*) and generate a severe oxidative stress including reactive oxygen species (ROS) *via* complex biochemical mechanisms (*3*) while the formed ROS in turn can accelerate the rate of AGEs formation (*8,9*). In addition, ROS can stimulate nuclear factor kappa B (NF-κB) to release pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) which play major roles in inflammatory processes (*10*). Phenolic substances from several plants have been reported to inhibit oxidative stress, glycation, or inflammation, however, no compound has all of these pharmacological effects simultaneously (*11–14*). The search for active compounds with multiple targets simultaneously remains challenging for the treatment of these severe chronic diseases. In addition, most reported plants

are inedible and present some toxic for human life (*15,16*). The intake of edible plant extracts should be a much better approach for ones seeking safe and potent bioactive compounds.

In this study, 4 species of edible plants, *Caesalpinia mimosoides*, *Zingiber officinale*, *Spondias dulcis*, and *Dolichandrone serrulata* were compared for inhibitory activities against oxidative stress, glycation, and inflammation. These four plants are commonly used as raw materials in Thai folk medicine. The ethanol extracts from commonly used parts of these plants were investigated. The most active plant extract was selected for constituent analysis using a high-performance liquid chromatography (HPLC).

2. Materials and Methods

2.1. Materials

Bovine serum albumin (BSA), ribose, sodium benzoate, aminoguanidine, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), butylated hydroxytoluene, quercetin, gallic acid, syringic acid, *p*-coumaric acid, ellagic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and lipopolysaccharides (LPS) obtained from *Escherichia coli* (serotype 0111:B4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu reagent, dimethyl sulfoxide (DMSO), sodium carbonate, ribose, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and potassium persulfate, were obtained from Merck (Darmstadt, Germany). Ethanol (EtOH), acetic acid, and hydrochloric acid were obtained from RCI Labscan Limited (Bangkok, Thailand). Fetal bovine serum (FBS), complete Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin-streptomycin, and 2-mercaptoethanol were obtained from Gibco BRL Life Technologies (Gaithersburg, MD, USA). AlamarBlue cell viability reagent was obtained from Invitrogen (Merelbeke, Belgium). Human monocytic leukemia (THP-1 cells) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

2.2. Plants and preparation of plant extracts

Fresh plant materials from four plant species (Table 1) were collected or purchased from the northern area of Thailand during July and August 2020. The used parts of each plant were thoroughly washed and cut into small pieces and then dried at 50°C for 24 h. The dried plant materials were pulverized and then macerated with 95% EtOH at room temperature for 48 h. The macerated mixture was subsequently filtered through Whatman No.1 filter paper (GE HealthCare Technologies, Chicago, IL, USA). The residue from filtration was further macerated and filtered in the same manner two more times. The filtrates of each plant material from these three macerations were pooled together and evaporated using a rotary evaporator (Eyela, Tokyo, Japan) until the solvent was completely removed. The obtained crude extracts were transferred into a tight container and stored at 4°C until use.

Table 1. Plant species and their part used

Scientific name	Part used
Caesalpinia mimosoides	Leaf
Zingiber officinale	Rhizome
Spondias dulcis	Stem bark
Dolichandrone serrulata	Leaf

2.3. Determination of total phenolic content

The total phenolic content of the obtained extracts was determined using Folin-Ciocalteu assay described by Sato *et al.* (*17*) with minor modifications. Briefly, the extract was dissolved in EtOH to yield a stock solution of 1 mg/mL. An aliquot of 20 µL this solution was mixed with 45 µL of Folin-Ciocalteu reagent for 2 min, followed by the addition of 135 µL of 20 mg/mL sodium carbonate. Next, the mixture was incubated for approximately 1 h at room temperature. Subsequently the absorbance at 750 nm was measured using microtiter plate reader (BioTek Instruments, Winooski, VT, USA). Gallic acid was used for calibration (10-500 µg/mL in EtOH). The total phenolic content is expressed as gallic acid equivalent (GAE) in mg of gallic acid to 1 g of the extract.

2.4. Determination of antioxidant activity

Free radical scavenging assay was performed according to a method previously described (*18*) with some modifications. Briefly, ABTS and potassium persulfate were dissolved separately in deionized water. The free radical of ABTS was generated by mixing 8 mL of 7 mM ABTS solution with 12 mL of 2.45 mM potassium persulfate solution. The obtained mixture was incubated in the dark at room temperature for 16 h. Then, EtOH was added to the mixture to obtain an absorbance of 0.7 at 750 nm. Stock solutions of the extracts were prepared by dissolving the crude extracts in EtOH at a concentration of 0.5 mg/mL. Then, an aliquot of 20 µL of this ethanol solution was mixed with 180 µL of the ABTS free radical solution. The mixture was incubated in the dark for 5 min at room temperature. Subsequently, the absorbance at 750 nm was measured using a microtiter plate reader (BioTek Instruments). Trolox was used for calibration. Quercetin and butylated hydroxytoluene at 1 mg/mL in EtOH were used as positive controls. Various concentrations (50-500 µM) of Trolox in EtOH were used for calibration. The results are expressed as Trolox equivalent

antioxidant capacity (TEAC) in mM of Trolox which had antioxidant capacity equivalent to 1 mg of the extract. Four standard phenolic compounds (gallic acid, syringic acid, *p*-coumaric acid, and ellagic acid) were investigated for their antioxidant activity using this assay. The IC_{50} value of each standard compound was calculated using GraphPad Prism software version 8.0.1.

2.5. Determination of antiglycation activity

Antiglycation activity of the samples was investigated by determining the inhibition of AGEs formation using BSA-ribose assay as previously described (*19*) with some modifications. Briefly, 10 mg/mL BSA, with or without 0.5 M ribose (and 0.008% sodium benzoate as a preservative), the plant extract in EtOH (0.1 mg/mL) and phosphate buffer (50 mM, pH 7.4) were mixed. The mixtures without extract and with or without ribose were used as negative controls. The reaction mixtures were incubated for 3 days at 45°C. Subsequently, the fluorescence intensity was measured using spectrofluorometer (Molecular Device, San Jose, CA, USA) at excitation and emission wavelengths of 370 and 440 nm, respectively. Aminoguanidine at the same concentration of the extracts (0.1 mg/mL) was used as a positive control. The percentage of AGEs inhibition is calculated using the following equation: AGEs inhibition (%) = $[(F_C - F_{CB}) - (F_S - F_{SB})/(F_C - F_{CB})] \times 100\%$. In which F_C and F_{CB} are the fluorescence intensities of the negative controls with and without ribose, respectively. F_S and F_{SB} are the fluorescence intensities of samples containing the extracts or aminoguanidine with and without ribose, respectively.

2.6. Determination of anti-inflammatory activity

2.6.1. Cell cultures

THP-1 cells were maintained according to ATCC recommendations. The cells were cultured at 37°C under 5% CO_2 in complete RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin and 0.05 mM 2-mercaptoethanol for 72 h before the experiments were performed.

2.6.2. Cytotoxicity on THP-1 cells

The possible cytotoxic effect of the obtained plant extracts on THP-1 cells was determined by colorimetric alamarBlue assay according to the manufacturer's protocol (Invitrogen, Merelbeke, Belgium) with slight modifications. Briefly, the concentration of THP-1 cells in the suspension was adjusted to 40×10^4 cells/mL using a hemocytometer for cell counting. Then, 100 µL of this cell suspension was seeded into each well of 96-well plates and incubated at 37°C, 5% CO_2 for 24 h. The extracts were dissolved in DMSO to obtain a concentration of 50 mg/mL, and then diluted with complete RPMI 1640 medium to obtain final concentrations of 0-0.5% v/v. These suspensions were added into the wells containing cells and further incubated in 5% CO_2 at 37°C for 24 h. Cell suspensions without extract or DMSO were used as negative controls. After 24 h, the cells originally in suspension were attached to the surfaces of the wells. An aliquot of 100 µL of culture medium was removed, and 10 µL of 10-fold alamarBlue solution was added to the wells. The cells were further incubated in the dark for 3 h. Subsequently, the plate was read at 562 nm and 600 nm as a reference wavelength using a microtiter plate reader (BioTek Instruments). The percentage of cell viability was calculated using the following equation: Cell viability (%) = $[(OD_{\text{test well}}) / (OD_{\text{negative control well}})] \times 100\%$. In which OD is the optical density and percentage of cell viability of the negative control is defined as 100%.

2.6.3. Effect of the extracts on inflammatory response

In this experiment, an aliquot of 200 µL of THP-1 cell suspension at a concentration of 40×10^4 cells/mL was seeded into each well of 24-well plates and incubated at 37°C in 5% CO_2 for 1 h. The extracts were dissolved in DMSO to yield a stock solution. This solution was diluted in complete RPMI 1640 medium to obtain a series of nontoxic concentrations to the cells. Then 300 µL of these dilutions or 0.12% DMSO in complete RPMI 1640 medium were added to the cell suspension and further incubated for 4 h at 37°C in 5% CO_2. After that, LPS was added to obtain a final concentration of 1 µg/mL and further incubated at 37°C in 5% CO_2 for 24 h. Next, the media were collected and centrifuged at 6,000 rpm for 3 min to remove non-attached cells. The supernatant was subsequently analyzed for interleukin-6 (IL-6) using an enzyme-linked immunosorbent assay (ELISA). The cells that were not treated with LPS served as a negative control and that were not pretreated with the extracts but incubated with LPS served as a positive control. IL-6 in the cell supernatants (100 µL) was determined by ELISA according to the manufacturer's protocol (BioLegend, San Diego, CA, USA). The optical density of the samples at 450 nm, corrected by the reference wavelength 562 nm, was measured using a microtiter plate reader (BioTek Instruments). Percentage of IL-6 secretion was calculated using the following equation: IL-6 secretion (%) = $[IL\text{-}6_{SAM}/IL\text{-}6_{LPS}] \times 100\%$. In which $IL\text{-}6_{SAM}$ represents the concentrations of the secreted IL-6 from THP-1 cells treated with the extracts or DMSO or untreated with LPS (negative control) and $IL\text{-}6_{LPS}$ represents the concentrations of the secreted IL-6 from the positive control. The percentage of IL-6 secretion of the positive control is defined as 100%.

2.7. HPLC analysis

The selected potential plant extract was analyzed using HPLC Shimadzu L2030 model (Kyoto, Japan) and a reversed phase Eurospher 100, C18 column, 4 mm i.d. × 250 mm, Knauer (Berlin, Germany). The system was conducted with a gradient program as previously described (*20*) with some modifications. Briefly, a gradient eluent composed of 1% acetic acid in water (A) and methanol (B) was used. The gradient program started from 100% of A for 1 min then the ratios of eluent A:B were changed to 70:30 and 40:60 at 10 and 20 min, respectively. After that, the composition of the eluent was put back to 100% of A at 25 min and held on for 10 min. The HPLC condition was operated with an injection volume of 10 µL, a mobile flow rate of 1 mL/min, and running time of 35 min. The eluent was monitored with UV/visible detector at a wavelength of 280 nm. Four standard phenolic compounds, gallic acid, syringic acid, *p*-coumaric acid, and ellagic acid were used as standard solutions for the quantification of phenolic compounds.

2.8. Statistical analysis

All experiments were carried out in triplicate. The results are expressed as mean values ± S.D. To determine statistical different between means ($p < 0.05$), ONE-WAY ANOVA and Tukey's Multiple tests were calculated using SPSS statistical software package v.17.0.

3. Results

3.1. Total phenolic content

The total phenolic content of the ethanol extracts obtained from the four different plants are expressed as GAE value as shown in Figure 1. *C. mimosoides* demonstrated the highest total phenolic content ($p < 0.05$) with a GAE value of 301 ± 8 mg/g, followed by *Z. officinale*, *S. dulcis*, and *D. Serrulata* with the GAE values of 187 ± 4, 159 ± 5, and 43 ± 2 mg/g, respectively.

3.2. Antioxidant activity

The antioxidant activities of the four extracts investigated by ABTS assay were calculated and expressed as TEAC values and shown in Figure 2. It was found that the highest free radical scavenging activity ($p < 0.05$) was obtained from the extract of *C. mimosoides* with a TEAC value of 5.9 ± 0.1 mM/mg, followed closely by that of *Z. officinale* with a TEAC value of 5.4 ± 0.2 mM/mg. The extracts of *S. dulcis* and *D. serrulata* showed low TEAC values of

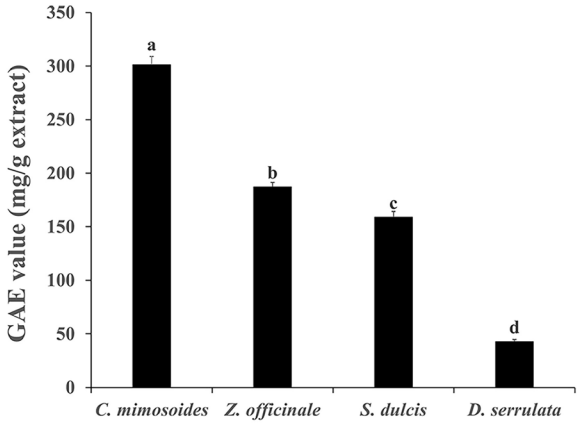

Figure 1. Total phenolic content of four plant extracts. Data represent mean ± SD of three independent experiments. Lowercase letters indicate significant difference between groups ($p < 0.05$).

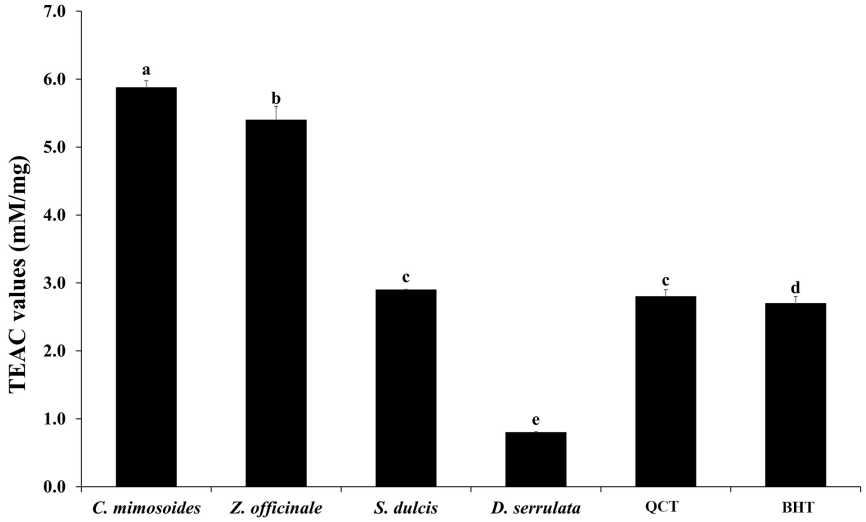

Figure 2. Antioxidant activity of four plant extracts compared to two positive controls, quercetin (QCT) and butylated hydroxytoluene (BHT). Data represent mean ± SD of three independent experiments. Lowercase letters indicate significant difference between groups ($p < 0.05$).

2.9 ± 0.1 and 0.8 ± 0.1 mM/mg, respectively, indicating that the antioxidant activity of these two extracts were significantly less than half of *C. mimosoides*. In addition, the TEAC values of quercetin and butylated hydroxytoluene were 2.8 ± 0.1 and 2.7 ± 0.1 mM/mg, respectively, indicating that the scavenging activity of the two positive controls was significantly lower than *C. mimosoides*.

3.3. Antiglycation activity

In the present study, the antiglycation activity of the extracts was investigated using the BSA-ribose assay. The activity was compared when the final concentration of the extracts and the positive control was the same (0.1 mg/mL). The results are expressed as the percentage of AGEs inhibition as demonstrated in Figure 3. *C. mimosoides* and *S. dulcis* extracts showed the same potential of AGEs inhibition of 11.4 ± 1.1% and 11.5 ± 0.5%, respectively, while that of *Z. officinale* and *D. serrulata* extracts were 5.2 ± 0.4% and 0.5 ± 0.8%, respectively. These results indicate that the AGEs inhibition activity of *C. mimosoides* and *S. dulcis* extracts was significantly ($p < 0.05$) higher than *Z. officinale* and *D. serrulata* extracts. In addition, aminoguanidine showed the inhibition power of only 3.1 ± 0.7%. These results indicate that *C. mimosoides* and *S. dulcis* extracts possessed AGEs inhibition activity approximately 3-times higher than the positive control.

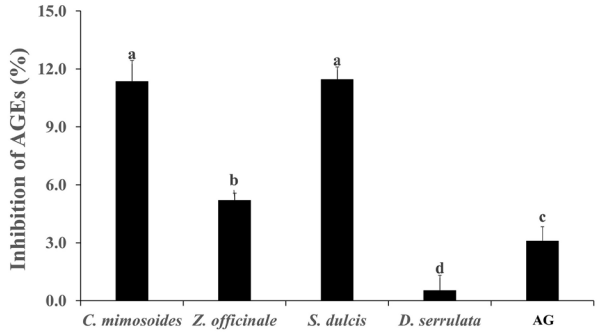

Figure 3. Antiglycation of four plant extracts compared to a positive control, aminoguanidine (AG). Data represent mean ± SD of three independent experiments. Lowercase letters indicate significant difference between groups ($p < 0.05$).

3.4. Anti-inflammatory activity

The results from cytotoxicity study indicated that the extracts from different plants possessed different levels of toxicity to THP-1 cells as shown in Figure 4. *C. mimosoides* and *S. dulcis* extracts were found to be nontoxic to THP-1 cells at all concentrations used. The extracts of *Z. officinale* and *D. serrulata* showed some toxicity to THP-1 cells, depending on the concentration used. The higher the concentration, the higher the cytotoxicity. Viable cells after exposure to *Z. officinale* extract at the concentrations of 125 and 250 µg/mL were 66 ± 7.8% and 52 ± 5.7%, respectively, while that after exposure to *D. serrulata* extract at these concentrations were 85 ± 8.1% and 65 ± 9.5%, respectively. The concentrations of all extracts at 60 µg/mL or less were selected for anti-inflammatory activity test. To access the effects of the four plant extracts on THP-1 cells, the cells were stimulated with 1 µg/mL LPS in the absence and presence of the extracts. The results as shown in Figure 5 demonstrate that large amounts of IL-6 (100%) were secreted from the cells upon LPS stimulation, whereas very small amounts of IL-6, near 0%, were observed in the LPS-unstimulated cells. DMSO, used as a solvent for the extracts, showed no effect on IL-6 secretion. The extracts of *C. mimosoides* and *Z. officinale* exhibited extremely potent anti-inflammatory activity in a concentration-dependent

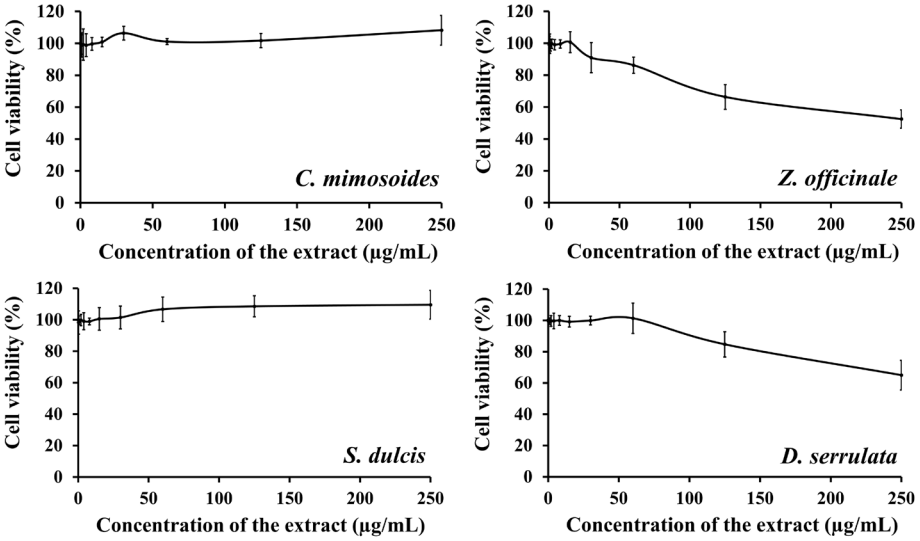

Figure 4. Cytotoxicity of four plant extracts against THP-1 cells. Data represent mean ± SD of three independent experiments.

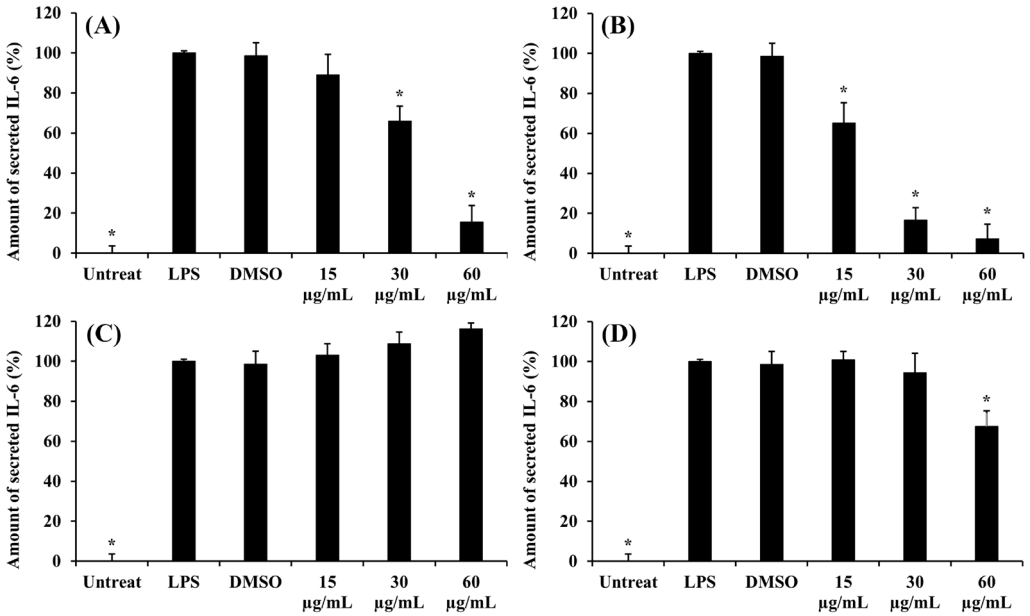

Figure 5. Anti-inflammatory activity of four plant extracts. Data represent mean ± SD of three independent experiments. Significant differences ($p < 0.05$) in comparison with those treated with LPS are indicated by *.

manner. The amount of IL-6 secreted from LPS-treated THP-1 cells was only 15 ± 8.3% and 7 ± 7.2% after the cells were exposed to those two extracts, respectively. This result indicates that both extracts inhibited IL-6 secretion by more than 85%. The inhibitory activity of *D. serrulata* extract also followed a dose dependent manner, but its inhibition power was significantly less than *C. mimosoides* and *Z. officinale* extracts. *S. dulcis* extract at all test concentrations did not inhibit IL-6 secretion. In addition, this extract tended to increase IL-6 secretion.

3.5. HPLC analysis

The above results suggested that *C. mimosoides* extract possessed the highest potential among all tested activities, so this extract was selected for HPLC analysis. Figure 6 shows the HPLC chromatogram of *C. mimosoides* extract in comparison with 4 standard phenolic compounds; gallic acid, syringic acid, *p*-coumaric acid, and ellagic acid. Comparing the retention times of these four standards (Figure 6A) with the extract (Figure 6B), it was observed that the peaks no. 1, 2, 3, and 4 of the extract seemed to be gallic acid, syringic acid, *p*-coumaric acid, and ellagic acid, respectively. To confirm this result, the extract was spiked with standard gallic acid. As expected, the peak height of peak no. 1 was obviously increased as seen in Figure 6C confirming that this peak belongs to gallic acid. Syringic acid, *p*-coumaric acid, and ellagic were added to the extract separately to identify the compounds presented in peaks no. 2, 3, and 4, respectively. The results as shown in Figure 6D, 6E, and 6F, respectively demonstrated that the peak height of peaks no. 2, 3, and 4 were increased accordingly. From this study, our results confirmed that gallic acid, syringic acid, *p*-coumaric acid, and ellagic acid were presented in *C. mimosoides* extract. Quantitative analysis indicated that *C. mimosoides* extract contained approximately 13.48%, 7.86%, 4.16%, and 7.63% of gallic acid, syringic acid, *p*-coumaric acid, and ellagic acid, respectively. From these results, it was considered that high activity of *C. mimosoides* extract might come from these compounds. To confirm this consideration, antioxidant activity of these four compounds was investigated. The results expressed as the percentage of free radical inhibition are demonstrated in Figure 7. From this result, the concentration of each compound that can inhibit 50% of free radicals (IC_{50}) was determined. It was found that among four tested compounds, gallic acid showed the highest antioxidant activity with the lowest IC_{50} value of 1.5 ± 0.1 µg/mL, followed closely by ellagic acid and syringic acid with IC_{50} values of 3.0 ± 0.1 µg/mL and 12.6 ± 0.1 µg/mL, respectively. The lowest antioxidant activity was obtained from *p*-coumaric acid, showing an IC_{50} value of 1.7 mg/mL.

4. Discussion

Oxidative stress, glycation, and inflammation are the major causes of several severe diseases such as cancer, diabetes, and cardiovascular disorders. Many attempts have been made to prevent the processes of these reactions and nowadays, people are interested in prevention using herbal medicines. *C. mimosoides* is a climbing shrub belonging the Fabaceae family and widely grows in various countries such as China,

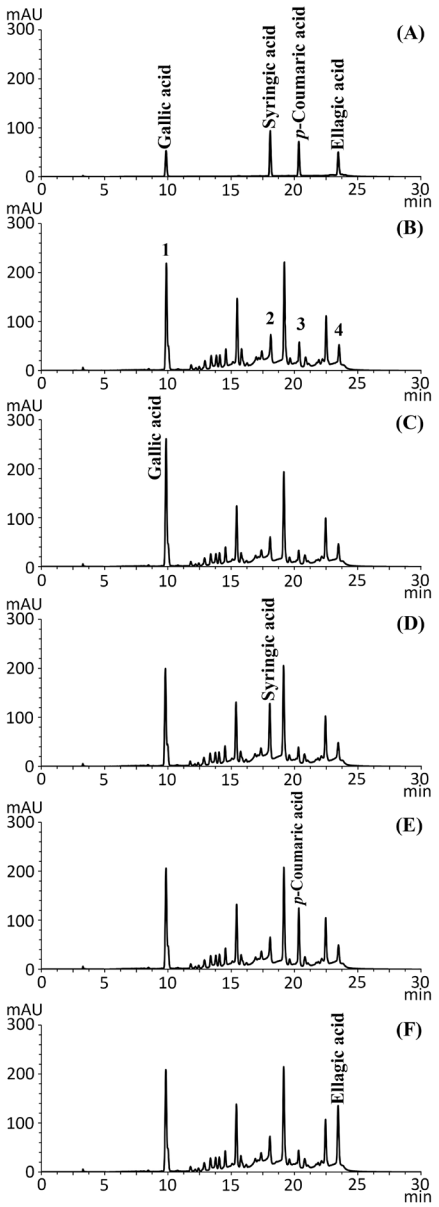

Figure 6. HPLC chromatograms of standard phenolic compounds (A), *C. mimosoides* extract (B), and *C. mimosoides* extract simultaneously spiked with gallic acid (C), syringic acid (D), (*p*-coumaric acid (E), and ellagic acid (F).

Myanmar, India, as well as in northern and north-eastern part of Thailand (*21*). Young shoots and leaves are consumed as vegetable and are traditionally used as a carminative and a remedy against dizziness and skin diseases (*22*). The plant has been reported to provide antioxidant (*23*), antimicrobial (*24*), anti-inflammatory (*25*), anticancer (*26*), and anti-diabetic activities (*27*). In the present study, the antioxidant, antiglycation, and anti-inflammatory activities of *C. mimosoides* were compared with that of *Z. officinale*, *S. dulcis*, and *D. serrulata*. These three plants are also edible and used in traditional medicines for various ailments associated with oxidation, glycation, and inflammation (*28–31*).

Phenolic compounds are good electron donors due to their structures which consist of an aromatic ring with hydroxyl group. Thus, phenolic compounds play pivotal role in antioxidant activity (*32*). Determination of total phenol content in plant extracts is a preliminary step in determining the antioxidant potential. The present study shows that among the four plant ethanol extracts, *C. mimosoides* extract contained the highest concentration of phenolic compounds. This finding is in line with the previous report showing that *C. mimosoides* had the highest phenol content among 33 edible plants (*33*). Furthermore, *C. mimosoides* extract exhibited the highest antioxidant activity *via* free radical scavenging mechanism. While the other three extracts, *Z. officinale*, *S. dulcis* and *D. serrulata* had significantly lower antioxidant activity with lower phenolic contents than *C. mimosoides* extract. These data confirm that phenolic compounds in plant extracts play an important role in antioxidant activity. Our data support the results from various authors. For example, previous report on several phenolic compounds in the leaves and fruits of *S. dulcis* exhibited strong antioxidant activity (*30*) and the previous report on linear relationship between the antioxidant activity and total phenolic content of the citrus fruit extracts (*34*). When *Z. officinale* and *S. dulcis* were compared, the antioxidant activity of *Z. officinale* extract was much higher than that of *S. dulcis*, although the phenolic content of *Z. officinale* extract was only slightly higher than that of *S. dulcis*. This result was considered that the antioxidant activity of plant extracts was not limited to phenolic compounds. The antioxidant activity may also come from the other secondary metabolites contained in plants, such as vitamins, alkaloids, and volatile oils (*35,36*).

AGEs are harmful products that occur in the late stage of glycation. Although several synthetic AGEs inhibitors have achieved promising advances *in vitro* and *in vivo*, but those synthetic compounds also possess dangerous side effects to humans. Therefore, the claims for natural or herbal substances are increasing. In the present study, we compared AGEs inhibition activity of extracts from four Thai medicinal plants using the BSA-ribose assay. As our findings showed that *C. mimosoides* possessed the highest level of antiglycation activity (with the highest phenol content and antioxidant activity), meanwhile, *D. serrulata* leaf extract showed very low antiglycation and antioxidant activities with low phenol content. This can be considered that phenolic compounds of *C. mimosoides* play a role in antiglycation activity due to antioxidation mechanism. Our results were consistent with the results of previous studies which showed a strong relationship between antiglycation and antioxidant properties of plant extracts (*37*). However, *Z. officinale* extract, which had higher phenolic content than *S. dulcis* extract possessed lower antiglycation activity than *S. dulcis* extract. This result suggests that the antiglycation activity of plant extracts may not be ascribed solely by their antioxidant activity or total phenolic content.

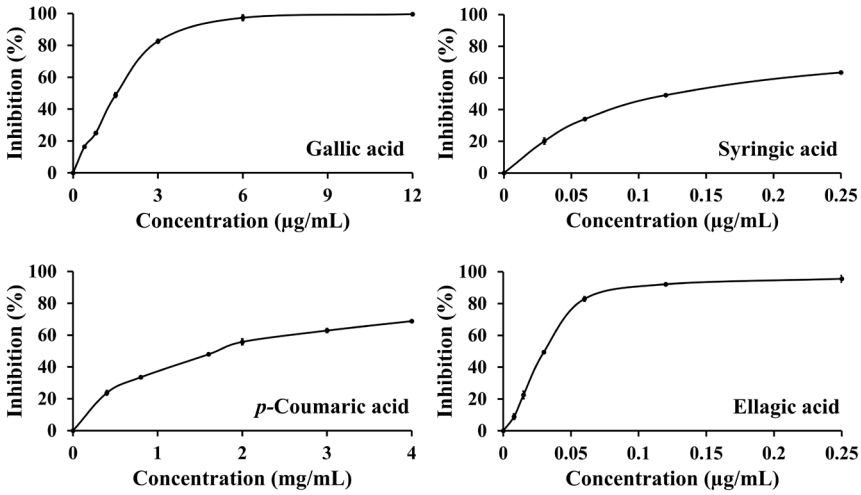

Figure 7. Free radical inhibition of four phenolic compounds presented in *C. mimosoides* extract. Data represent mean ± SD of three independent experiments.

IL-6 is one of pro-inflammatory cytokines that secretes from leukocytes when the cells are stimulated by stimulants such as bacterial LPS. In the present study, we investigated inhibitory effect of the four plant extracts on IL-6 secretion from LPS-stimulated THP-1 cell, the human leukemia monocytic cell line. In this study, the non-toxic concentration (cell viability above 80%) of the extracts was selected based on cell cytotoxicity experiments. It was found that the extracts of *C. mimosoides* and *Z. officinale* showed significant inhibitory effect on IL-6 secretion especially at concentrations of 30 and 60 µg/mL. Our findings supported the data from other reports that phenolic compounds influence anti-inflammatory activities (*38*). The previous studies reported that the anti-inflammatory activity of *Z. officinale* was from several terpenoids including gingerol (*39*). Our results also demonstrate that *D. serrulata* extract tended to inhibit IL-6 secretion at concentrations of 60 µg/mL whereas *S. dulcis* extract had no potential to reduce IL-6 secretion at all tested concentration. The results of this finding show that not only phenol compounds but also other chemical constituents that may be related to the anti-inflammatory process.

Among the four plant ethanol extracts, *C. mimosoides* extract was considered as the most potential extract for inhibition of oxidation, glycation, and inflammation because it has the highest antioxidant and antiglycation activities. This extract also has high anti-inflammatory activity without cytotoxicity. Previous studies have reported that *C. mimosoides* is high in phenolic compounds, vitamins, and carotenoids (*33*). This led us to further analyze the chemistry of this extract. Results performed using HPLC confirmed the presence of four main phenolic compounds: gallic acid, syringic acid, *p*-coumaric acid, and ellagic acid. This result supports previous studies reporting gallic acid and *p*-coumaric acid as the main phenolic compounds found in *C. mimosoides* extract (*21,23,26,27*). Our findings shed new light on syringic acid and ellagic acid in *C. mimosoides* extracts that were not previously reported. In addition, our results show the presence of very low IC_{50} values in free radical scavenging activity of gallic acid, syringic acid, and ellagic acid. This suggests that these phenolic compounds possess very high antioxidant activity and play a pivotal role on the antioxidant activity of *C. mimosoides* extract. As free radicals mainly can enhance glycation and inflammation, it was considered that the high antioxidant activity can lead to the high inhibition of glycation and inflammation. Although further studies of antiglycation and anti-inflammation of these compounds are required.

5. Conclusion

The present study shows a comparative investigation on the antioxidant, antiglycation, and anti-inflammatory activities present in the ethanol extracts of four edible and medicinal plants. All extracts contain phenolic compounds but in different levels. The antioxidant, antiglycation, and anti-inflammatory activities of some plant extracts are not ascribed solely by phenolic compounds, but it is likely affected by other metabolites existed in the extracts. Among these extracts, *C. mimosoides* extract possesses the highest antioxidant and antiglycation activities. It also possesses high anti-inflammatory activity without any cytotoxicity. The main phenolic compounds found in this extract are gallic acid, syringic acid, *p*-coumaric acid, and ellagic acid. We are the first to report that syringic acid and ellagic acid are the phenolic compounds in *C. mimosoides*. It is concluded that *C. mimosoides* extract might be a promising natural source for treatment

of chronic non-communicable diseases caused by oxidative stress, glycation, and inflammation.

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**Address correspondence to:*
Siriporn Okonogi, Research Center of Pharmaceutical Nanotechnology, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail: okng2000@gmail.com

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Original Article

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University students' living conditions during the COVID-19 pandemic and predictors of their subjective health views: A cross-sectional survey

Kiyoshi Hotta[1], Yukihiro Mori[1,2], Makoto Aoike[2], Yukihiro Shigeno[3], Yuka Aoyama[2,4], Mamoru Tanaka[5], Hana Kozai[5], Hatsumi Kawamura[2], Masato Tsurudome[2,6], Morihiro Ito[2,6,*]

[1] Center for Nursing Practicum Support, Chubu University, Aichi, Japan;
[2] Graduate School of Life and Health Sciences, Chubu University, Aichi, Japan;
[3] The Fire Department Headquarters in Kasugai-City, Aichi, Japan;
[4] Department of Clinical Engineering, College of Life and Health Sciences, Chubu University, Aichi, Japan;
[5] Department of Food and Nutritional Sciences, College of Bioscience and Biotechnology, Chubu University, Aichi, Japan;
[6] Department of Biomedical Sciences, College of Life and Health Science, Chubu University, Aichi, Japan.

SUMMARY This study aimed to explore the factors influencing subjective health views based on the living conditions and concerns of university students during the coronavirus infection 2019 (COVID-19) pandemic. From March to April 2021, a questionnaire survey was administered to 8,547 Japanese university students, and logistic regression analysis was used to explore factors related to subjective health views. The results showed that satisfaction with quality of sleep (OR = 2.651, 95% Cl 2.370-2.966, $p < 0.001$), satisfaction with university life (OR = 2.486, 95%Cl 2.215-2.789, $p < 0.001$), satisfaction with diet (OR = 1.849, 95% CI: 1.496-2.285, $p < 0.001$), regular exercise (OR = 1.759, 95% CI: 1.594-1.941, $p < 0.001$), consciousness of nutritional balance (OR = 1.276, 95% CI: 1.147-1.420, $p < 0.001$), eating breakfast every day (OR = 1.247, 95% CI: 1.121-1.387, $p < 0.001$), and consuming soft drinks at least once a week (OR = 0.865, 95% CI: 0.755-0.966, $p = 0.010$) were positive factors for subjective views of health. On the other hand, anxiety about whether the necessary credits can be obtained (OR = 0.885, 95% CI: 0.799-0.980, $p = 0.019$), infection from minimal outings (OR = 0.881, 95% CI: 0.794-0.976, $p = 0.016$) building and maintaining friendships on campus (OR = 0.867, 95% CI: 0.767-0.980, $p = 0.023$), and being able to continue working (OR = 0.713, 95% CI: 0.640-0.795, $p < 0.001$) were identified as negative factors. To ensure a healthy university life during the COVID-19 pandemic or future pandemic, supports tailored to students' living conditions and measures to address their anxieties are required.

Keywords COVID-19 pandemic, university students, subjective health views

1. Introduction

Novel coronavirus infection (COVID-19) was first confirmed in Wuhan, China, in December 2019 (*1*). As at March 10, 2021, 118,496,719 confirmed cases of COVID-19, including 2,726,603 deaths were reported globally. The highly contagious nature of this infection required preventive measures such as wearing masks, physical distancing (*2*), regular washing of hands, use of sanitizers, as well as immediate quarantine and lockdown, all of which are measures that had brought about substantial lifestyle changes and global consequences for students at all levels (*3*). As part of ongoing efforts to further limit the person-to-person transmission of the disease, educational institutions have had to shift to online learning programs. As expected, since not all educators and students were prepared for this sudden change, many did not have adequate access to the appropriate infrastructures and resources. This change in the mode of education has created new and unanticipated challenges for many students (*4*), and in some cases, it has dramatically disrupted the educational process. Many students in tertiary institutions were forced to stay at home and learn the required material on their own. Moreover, they had limited access to learning resources and little or no opportunity for personal

interaction with teachers or classmates. One recent study reported that this situation has led to considerable stress among university students (*4*). Other recent studies revealed increased loneliness, anxiety, and depression due to lockdown, movement restriction, and transition to online learning program during the COVID-19 pandemic (*5,6*). The above sources of stress can have an undesirable impact on learning and may lead to mental health problems (*7*), especially among undergraduate and postgraduate university students, who are the target respondents in this study.

While it is important for different universities to find ways of addressing mental health issues such as anxiety and stress experiences of students during the COVID-19 pandemic (*8*), most universities do not have the infrastructures or resources required to facilitate effective action regarding mental health support for students in these situations. In addition, many universities did not employ specialized mental health counselors during the period (*4*). Ideally, stakeholders of universities should be aware of mental health issues, especially for students who are suffering from mental distress as a result of restrictions or lockdown during the COVID-19 pandemic, and should devise appropriate measures for responding to such situations. Appropriate response actions (*8,9*) can help students to live healthy lives as well as to support their wellbeing, motivate them to study, facilitate effective learning among them during and after the pandemic, and provide emotional stability. Furthermore, most university students in Japan are working adults after graduation and are the leaders of the next generation. However, at the time, research on Japanese university students who experienced the COVID-19 pandemic for one year focused on specific university students, such as depression measurement and nursing students, and there were no comprehensive studies of the situation of students attending comprehensive universities. Therefore, the purpose of this study was to understand the living conditions of university students and to find out the factors influencing their subjective health perceptions one year after the COVID-19 pandemic. We hypothesized that changes in life and anxiety during the COVID-19 pandemic would be influential factors in predicting Japanese university students' subjective views of health.

2. Materials and Methods

2.1. Respondents

This study adopted a cross-sectional study design. The target population of this study was 8,547 undergraduate and postgraduate students of University A. University A is a general university with about 10,000 students, most of them are from different regions in Japan. The survey was conducted from March to April 2021 using a self-administered, anonymous, questionnaire. The survey was conducted during the third to fourth waves of the COVID-19 pandemic in Japan when the state of emergency declaration by the government had been lifted.

2.2. Survey items

All respondents were asked about their sex, age, height, and weight as basic attributes. Data obtained from the respondents were analyzed to compare their basic attributes.

2.2.1. Subjective health views

The respondents were asked to choose between "healthy" and "not healthy" in terms of their subjective health views. Subjective health is defined in this study as a subjective perception of one's own health status, without distinguishing between physical, mental, and social aspects.

2.2.2. Surveys on daily living

The respondents were asked to answer the questions on 11 items that are related to their daily life using a two-response option. The respondents answered "satisfied" or "not satisfied" with their dietary habits, the quality of their sleep, and their university life. They also responded whether they were "aware" or "not aware" of the nutritional balance of foods they took; "substituted" or "not substituted" snacks for meals; "ate" or "did not eat" breakfast daily; "drank" or "did not drink" alcohols at least once in a week; "drank" or "did not drink" soft drinks at least once in a week; "consumed" or "did not consume" healthy foods; slept "more than 7 hours" or "less than 7 hours" a day; and responded "yes" and "no" for regular exercise.

2.2.3. Infection prevention behavior

Respondents were provided with "yes" or "no" options on the following 14 infection-prevention behaviors they are practicing: wearing a mask, washing hands, disinfecting hands, cough etiquette, gargling, avoiding crowds, frequent ventilation, avoiding short-distance conversations, getting enough sleep, gathering information through news, being conscious of their nutritional balance, disinfecting their surroundings, avoiding contact with mucous membranes such as eyes and nose, and using contact confirmation applications. The contact confirmation application (app) was developed by the Ministry of Health, labor, and Welfare and uses the proximity communication function of smartphones to receive notification of the possibility of contact with a positive person while ensuring privacy. The app was developed with the hope that users can receive support from health centers and other institutions

at an early stage by identifying the possibility of contact with an infected person, and that this will help to prevent the spread of infection.

2.2.4. Anxiety factors

The respondents were asked questions about their concerns on the COVID-19 pandemic using two response options of "yes" or "no" for the following 21 items, 6 of which were on lack of a cure, inaccessibility to Polymerase Chain Reaction (PCR) testing, the overwhelmed medical system, possible infection from minimal outings necessary for daily life, uncertainty about how long the COVID-19 pandemic will last, and the possibility of being criticized and discriminated against by others if they become infected. Ten items about student life were: learning and lectures at the university, job seeking, earning credits for graduation, exercises and practical training necessary for the future, participating in internships, acquiring more certificates, financial problems, maintaining and building friendships on campus, organizing university events, and participating in club and volunteer activities. Five items on their future and career path were: whether or not they would be able to find a job, whether or not they would be able to find the type of work they wanted, whether or not they would be able to find a job at the company of their choice, whether or not their place of employment would be stable, and whether or not they would be able to last long in a job.

2.3. Statistical analysis

Data collected were analyzed using descriptive statistics to check proportions. The students' differences in Gender and subjective health views were compared using Pearson's χ^2 test. One-way ANOVA (Games-Howell method) was used to compare the differences in age, height, weight, and BMI. Next, Pearson's χ^2 test was used to determine the differences between "healthy" and "not healthy" responses in the subjective view of health for 11 items of life, 21 items of anxiety, and 14 items of infection prevention behavior. The 21 items of anxiety were also subjected to Pearson's χ^2 test to confirm their association with grade level. Furthermore, a univariate analysis of each item was conducted to confirm the factors influencing subjective views of health, and "getting enough sleep" and "being conscious of nutritional balance" were excluded from the infection prevention behaviors, which overlapped in content. Binomial logistic regression analysis (forward selection (likelihood ratio)) was then conducted with the subjective health views as the dependent variable, and 11 items related to daily life, 12 items related to infection prevention behaviors, and 21 items related to anxiety as independent variables. The respondents' grade was also analyzed as an independent variable to adjust for the effect of grade. All analyses were conducted using SPSS statistics 26 (IBM Corp Armonk. NY. USA), and $p < 0.05$ was considered statistically significant.

2.4. Ethical considerations

Subjects were informed in writing of the purpose, methods, and management of personal information of this study, and their consent was obtained by having them complete a questionnaire. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Review Committee of Chubu University (approval number 20200095).

3. Results

3.1. Attributes of the target population

A total of 8,117 (95.0%) valid responses were obtained for the study, of which 5,551 (68.4%) were those from males. The percentages of the first-year, second-year, third-year, and fourth-year, and postgraduate students who reported a positive subjective view of health were 59.7%, 46.5%, 48.5%, 49.5%, and 43.2%, respectively. The percentages of second year, third year, and postgraduate students who reported good subjective health were significantly lower than that of the first-year students ($p < 0.001$). There was a significant difference in height between the first- and second-year students ($p = 0.039$). Weight was significantly different for all the first year ($p < 0.001$), second-year ($p < 0.001$), third-year ($p = 0.007$) and fourth-year ($p = 0.004$) compared to postgraduate students, with postgraduate students having a higher weight. Moreover, the first-year ($p = 0.001$) and second-year ($p = 0.001$) students had lower mean BMI values than postgraduate students (Table 1).

3.2. Perceptions and behaviors about life by subjective views of health

Table 2 shows the results of the respondents who responded that they were healthy in the subjective view of health. A total of 4,044 (96.5%) respondents were satisfied with their dietary habits; 3,129 (74.7%) were conscious of their nutritional balance; 2,987 (71.3%) ate breakfast every day; 4,126 (98.4%) replaced their meals with snacks; 796 (19.0%) drank alcohols at least once a week, and 3,026 (72.2%) drank soft drinks at least once a week; 673 (16.1%) consumed health foods at least once a week; 47.1% slept for 7 hours or more; 84.4% were satisfied with their subjective sleep quality; 2,126 (50.7%) exercised regularly, and finally, 3,756 (89.6%) were satisfied with their university life.

The "healthy" group was more likely than the "not healthy" group to answer in the affirmative in the following 8 items: satisfied with their diet, conscious of nutritional balance, eat breakfast every day, substitute

Table 1. Basic attributes of the subjects (*n* = 8,117)

Items	First-year students			Second-year student			Third-year student			Fourth-year student			Postgraduate students			*p*
n (%)	2,595	(32.0)		2,050	(25.3)		1,745	(21.5)		1,528	(18.8)		199	(2.5)		
Gender (Male: %)	1,819	(70.1)	*	1,375	(67.1)		1,229	(70.4)		978	(64.0)		150	(75.4)		< 0.001
Healthy	1,548	(59.7)	*	954	(46.5)	**	846	(48.5)	**	757	(49.5)		86	(43.2)	**	< 0.001
	Mean	*(SD)*		*Mean*	*(SD)*		*Mean*	*(SD)*		*Mean*	*(SD)*		*Mean*	*(SD)*		
Height	167.1	(8.23)		166.4	(8.53)		167.1	(8.33)		166.5	(8.94)		168.3	(8.22)		0.039
Body weight	59.3	(11.28)		58.6	(11.23)		60.3	(11.99)		60.1	(13.28)		63.3	(12.56)		< 0.05
BMI (Kg/m^2)	21.2	(3.26)		21.1	(3.38)		21.6	(3.38)		21.6	(3.87)		22.3	(3.78)		0.001

Gender was compared by the χ^2 test; *, cells with adjusted standardized residuals greater than or equal to +2.0; **, cells with adjusted standardized residuals greater than or equal to -2.0; Age, height, weight, and BMI; Body Mass Index were compared by one-way ANOVA: Games-Howell method.

Table 2. Cross-tabulation by subjective views of health (*n* = 8,117)

Items	Healthy			Not healthy			*p*
	n (%) 4,191 (51.6)			*n* (%) 3,926 (48.4)			
Satisfaction with eating habits	4,044	(96.5)	*	3,504	(89.3)		< 0.001
Consciousness of nutritional balance	3,129	(74.7)	*	2,405	(61.3)		< 0.001
Eating breakfast every day	2,987	(71.3)	*	2,359	(60.1)		< 0.001
Substituting meals with snacks	4,126	(98.4)	*	3,806	(96.9)		< 0.001
Drinking alcohols at least once/week	796	(19.0)		858	(21.9)	*	0.002
Drinking soft drinks at least once/week	3,026	(72.2)		2,987	(76.1)	*	< 0.001
Consumption of healthy foods	673	(16.1)		659	(16.8)		0.385
Sleeping at least 7 hours a night	1,976	(47.1)	*	1,523	(38.8)		< 0.001
Satisfaction with sleep quality	3,538	(84.4)	*	2,376	(60.5)		< 0.001
Regular exercise	2,126	(50.7)	*	1,311	(33.4)		< 0.001
Satisfaction with university life	3,756	(89.6)	*	2,978	(75.9)		< 0.001

χ^2 test, *, cells with adjusted standardized residuals greater than or equal to +2.0.

snacks for meals, sleep 7 or more hours, satisfied with sleep quality, exercise regularly, and satisfied with university life. The percentage of respondents who were satisfied with their university life was high (all $p <$ 0.001). Conversely, the "not healthy" group had higher percentages of those who drank alcohols and soft drinks at least once in a week than the "healthy" group (Table 2).

3.3. COVID-19 pandemic anxiety factors by subjective health views

The respondents were asked about their concerns about the COVID-19 pandemic, student life, and future and career paths (Table 3). The results showed that the "not healthy" group was more likely than the "healthy" group to be concerned about the following 20 items: "lack of a cure" (25.7%), "inaccessibility to PCR testing" (24.5%), "the overwhelmed medical system" (33.9%), " Infection by minimal outings for daily living" (34.4%), "how long the COVID-19 pandemic will continue (38.5%)", and "criticism and discrimination from others when infected" (35.7%). Regarding student life, respondents were concerned about "lectures" (59.2%), "job seeking" (54.7%), "earning credits for graduation" (42.9%), "conducting exercises and practical training" (40.8%), "internships" (33.5%), "acquiring more certificates" (30.0%), "financial problems" (28.0%), "maintaining and building friendships on campus" (25.2%) and "club activities and volunteer activities" (22.6%). As for the future, the respondents were concerned about whether they would be able to find a job (73.9%), get a job they wanted (52.8%), find a job at the company they wanted (35.6%), have a stable job (42.8%), and whether they would be able to last at a job (36.5%) (Table 3).

3.4. Infection prevention behavior

Table 4 shows the infection prevention behaviors currently practiced. Compared to the "healthy" group, the "not healthy" group showed lower implementation rates for the following six items: washing hands (78.2%), gargling (53.7%), avoiding short-distance conversations (21.7%), avoiding contact with mucous membranes such as the eyes and nose (41.6%), getting enough sleep

Table 3. Crosstabulation results of anxiety by subjective views of health (*n* = 8,117)

Items	Healthy			Not healthy			*p*
	n (%)			*n* (%)			
	4,191	(51.6)		3,926	(48.4)		
Anxiety about COVID-19							
Lack of a cure	928	(22.1)		1,008	(25.7)	*	< 0.001
Inaccessibility to PCR testing	865	(20.6)		, 962	(24.5)	*	< 0.001
The overwhelmed medical system	1,204	(28.7)		1,330	(33.9)	*	< 0.001
Infection by minimal outings for daily living	1,222	(29.2)		1,352	(34.4)	*	< 0.001
How long will the COVID-19 pandemic last	1,395	(33.3)		1,512	(38.5)	*	< 0.001
Criticism and discrimination from others	1,290	(30.8)		1,402	(35.7)	*	< 0.001
Anxiety about student life							
Whether lectures will be held	2,323	(55.4)		2,324	(59.2)	*	0.001
Job seeking	2,007	(47.9)		2,149	(54.7)	*	< 0.001
Earning credits for graduation	1,583	(37.8)		1,684	(42.9)	*	< 0.001
Conducting exercises/practical training	1,491	(35.6)		1,603	(40.8)	*	< 0.001
Internships	1,202	(28.7)		1,315	(33.5)	*	< 0.001
Acquisition of qualifications	1,089	(26.0)		1,179	(30.0)	*	< 0.001
Financial problems	950	(22.7)		1,098	(28.0)	*	< 0.001
Maintaining and building friendships on campus	799	(19.1)		989	(25.2)	*	< 0.001
University events	821	(19.6)		810	(20.6)		0.245
Club activities and volunteering activities	858	(20.5)		886	(22.6)	*	0.023
Concerns about employment and career paths							
Getting a job	2,798	(66.8)		2,901	(73.9)	*	< 0.001
Getting a desired job	2,007	(47.9)		2,074	(52.8)	*	< 0.001
Getting a job at a desired company	1,359	(32.4)		1,396	(35.6)	*	0.003
Stability of employment	1,477	(35.2)		1,679	(42.8)	*	< 0.001
Ability to last at a job	1,046	(25.0)		1,434	(36.5)	*	< 0.001

χ^2 test, *, cells with adjusted standardized residuals greater than or equal to +2.0.; COVID-19, coronavirus disease 2019; PCR, polymerase chain reaction.

Table 4. Infection prevention behaviors by subjective views of health (*n* = 8,117)

Items	Healthy			Not healthy			*p*
	n (%)			*n* (%)			
	4,191	(51.6)		3,926	(48.4)		
Wearing a mask	4,120	(98.3)		3,861	(98.3)		0.931
Using water (or basin, *etc*.) for washing hands	3,373	(80.5)		3,069	(78.2)	**	0.011
Gargling	2,579	(61.5)		2,110	(53.7)	**	< 0.001
Hand sanitization	3,173	(75.7)		2,972	(75.7)		0.992
Cough etiquette	2,328	(55.5)		2,215	(56.4)		0.434
Avoiding crowds	2,320	(55.4)		2,139	(54.5)		0.435
Avoiding short-distance conversations	1,027	(24.5)		850	(21.7)	**	0.002
Avoiding touching mucous membranes such as eyes and nose	1,099	(26.2)		890	(41.6)	**	< 0.001
Disinfection of surroundings	1,089	(26.0)		1,010	(25.7)		0.791
Frequent ventilation	1,907	(45.5)		1,735	(44.2)		0.237
Getting enough sleep	1,835	(43.8)		1,156	(29.4)	**	< 0.001
Consciousness of nutritional balance	1,366	(32.6)		895	(22.8)	**	< 0.001
Gathering information from the news, *etc*.	1,507	(36.0)		1,391	(35.4)		0.626
Contact Confirmation App	598	(14.3)	**	636	(16.2)		0.015

χ^2 test, **, items with adjusted residue coefficient less than or equal to -2.0; App, application.

(29.4%), and being conscious of nutritional balance (22.8%).

3.5. COVID-19 pandemic anxiety factors by grade level

Table 5 shows the percentages of respondents who answered "anxious" by grade. Among the items with low values for the first-year students were "lack of cure" (19.6%), "inaccessibility to PCR testing" (19.0%), "overwhelmed medical systems" (27.0%), "infection from outings" (27.8%), "how long it will last" (31.3%), "criticism from others when infected" (29.8%), "job seeking" (35.1%), and "participating in an internship" (21.7%). Others are whether they could find a job

Table 5. Cross-tabulation results of anxiety by grade level for each of the anxiety factors (n = 8,117)

Items	First-year students			Second-year student			Third-year student			Fourth-year student			Postgraduate students			p
	n (%)			n (%)			n (%)			n (%)			n (%)			
	2,595	(32.0)		2,050	(25.3)		1,745	(21.5)		1,528	(18.8)		199	(2.5)		
Anxiety about COVID-19																
Lack of a cure	509	(19.6)	**	1,549	(75.6)		1,284	(73.6)		1,112	(72.8)	*	150	(75.4)		< 0.001
Inaccessibility to PCR testing	493	(19.0)	**	476	(23.2)		421	(24.1)		398	(26.0)	*	39	(19.6)		< 0.001
Overwhelmed medical system	701	(27.0)	**	639	(31.2)		585	(33.5)		536	(35.1)	*	73	(36.7)		< 0.001
Infection from minimal outings for daily living	721	(27.8)	**	666	(32.5)		573	(32.8)		549	(35.9)	*	65	(32.7)		< 0.001
How long will the COVID-19 pandemic last	813	(31.3)	**	751	(36.6)		642	(36.8)		618	(40.4)	*	83	(41.7)		< 0.001
Criticism from others in case of infection	774	(29.8)	**	695	(33.9)		597	(34.2)		565	(37.0)	*	61	(30.7)		< 0.001
Anxiety about student life																
Whether lectures will be held	1,669	(64.3)	*	1,287	(62.8)	*	964	(55.2)		627	(41.0)	**	100	(50.3)		< 0.001
Job seeking	910	(35.1)	**	880	(42.9)	**	1,138	(65.2)	*	1,109	(72.6)	*	119	(59.8)	*	< 0.001
Earning credits	1,303	(50.2)	*	894	(43.6)	*	596	(34.2)	**	438	(28.7)	**	36	(18.1)	**	< 0.001
Conducting exercises and practical training	1,011	(39.0)		860	(42.0)		745	(42.7)		425	(27.8)	**	53	(26.6)	**	< 0.001
Participation in Internships	562	(21.7)	**	515	(25.1)	**	915	(52.4)	*	454	(29.7)		71	(35.7)		< 0.001
Qualifications (including driver's license)	826	(31.8)	*	633	(30.9)	*	516	(29.6)		268	(17.5)	**	25	(12.6)	**	< 0.001
Financial problems	640	(24.7)		539	(26.3)		474	(27.2)	*	336	(22.0)	**	59	(29.6)		0.003
Maintaining and building friendships on campus	750	(28.9)	*	587	(28.6)	*	243	(13.9)	**	181	(11.8)	**	27	(13.6)	**	< 0.001
Organizing University Events	700	(27.0)		540	(26.3)	*	236	(13.5)	**	128	(8.4)	**	27	(13.6)	**	< 0.001
Club activities, circles and volunteering activities	805	(31.0)	*	497	(24.2)	*	266	(15.2)	**	156	(10.2)	**	20	(10.1)	**	< 0.001
Anxiety about the future and career path																
Getting a job	1,649	(63.5)	**	1,459	(71.2)		1,302	(74.6)	*	1,163	(76.1)	*	126	(63.3)	**	< 0.001
Getting the desired job type	1,293	(49.8)		982	(47.9)		910	(52.1)		792	(51.8)		104	(52.3)		0.059
Getting a job at a desired company	717	(27.6)	**	636	(31.0)	**	683	(39.1)	*	640	(41.9)	*	79	(39.7)		< 0.001
Stability of the employment	918	(35.4)	**	833	(40.6)		759	(43.5)	*	575	(37.6)		71	(35.7)		< 0.001
Ability to last on a job	719	(27.7)	**	655	(32.0)		585	(33.5)	*	462	(30.2)		59	(29.6)		0.001

χ^2 test; *, cells with adjusted standardized residuals greater than or equal to +2.0; **, cells with adjusted standardized residuals greater than or equal to -2.0; COVID-19, coronavirus disease 2019; PCR, polymerase chain reaction.

Table 6. Results of binomial logistic regression analysis affecting subjective views of health (*n* = 8,117)

Items	B	symmetric digital subscriber line	OR	95% Cl		*p*
				low	high	
Items related to daily life						
Satisfaction with sleep quality	0.975	0.057	2.651	2.370	2.966	< 0.001
Satisfaction with university life	0.911	0.059	2.486	2.215	2.789	< 0.001
Satisfaction with diet	0.615	0.108	1.849	1.496	2.285	< 0.001
Regular exercise	0.565	0.050	1.759	1.594	1.941	< 0.001
Consciousness of nutritional balance	0.244	0.055	1.276	1.147	1.420	< 0.001
Eating breakfast every day	0.221	0.054	1.247	1.121	1.387	< 0.001
Drinking soft drinks at least once a week	-0.145	0.056	0.865	0.775	0.966	0.010
Items related to anxiety						
Whether or not you can earn credits	-0.122	0.052	0.885	0.799	0.980	0.019
Infection from minimal outings	-0.127	0.053	0.881	0.794	0.976	0.016
Maintaining and building friendships on campus	-0.143	0.063	0.867	0.767	0.980	0.023
Ability to last on a job	-0.338	0.055	0.713	0.640	0.795	< 0.001

Model χ^2 test $p < 0.001$, Hosmer-Lemeshow test $p = 0.003$, Discriminative accuracy 66.9.

(63.5%), whether they could find a job at their desired company (27.6%), whether their job would be stable (35.4%), and whether they would last on a job (27.7%). The items with low scores for the fourth-year students were lectures (41.0%), earning credits (28.7%), doing exercises and practical training (27.8%), acquiring more certificates (17.5%), financial problems (22.0%), maintaining and building friendships on campus (11.8%), holding university events (8.4%) and club activities (10.2%).

3.6. Factors influencing subjective views of health

A logistic regression analysis was conducted to determine the respondents' subjective health view as the dependent variable, 11 items related to life, 12 items related to infection prevention behaviors, 21 items related to anxiety, and grade as the independent variables. As show the Table 6, seven items related to daily life and four items related to anxiety were extracted as factors influencing the subjective view of health. No item related to infection prevention behavior was identified. The items related to life were "satisfaction with the quality of sleep" (OR = 2.651, 95% Cl 2.370-2.966, $p < 0.001$), "satisfaction with university life" (OR = 2.486, 95% Cl 2.215-2.789, $p < 0.001$), "satisfaction with diet" (OR = 1.849, 95% Cl 1.496-2.285, $p < 0.001$), "regular exercise" (OR = 1.759, 95% Cl 1.594-1.941, $p < 0.001$), "consciousness of nutritional balance" (OR = 1.276, 95% Cl 1.147-1.420, $p < 0.001$), "consuming breakfast every day" (OR = 1.247, 95% Cl 1.121-1.387, $p < 0.001$), and "consuming soft drinks at least once in a week" (OR = 0.865, 95% Cl 0.755-0.966, $p = 0.010$). Items related to anxiety were "whether the student will be able to obtain necessary credits" (OR = 0.885, 95% Cl 0.799-0.980, $p = 0.019$), "infection from minimal outings" (OR = 0.881, 95% Cl 0.794-0.976, $p = 0.016$), "maintaining and building friendships on campus" (OR = 0.867, 95% Cl 0.767-0.980, $p = 0.023$), and "whether the student would be able to last on a job" (OR = 0.713, 95% Cl 0.640-0.795, $p < 0.001$) (Table 6).

4. Discussion

In this study, we conducted a questionnaire survey to explore the living conditions of university students and to find out the factors influencing their subjective health perceptions one year after the COVID-19 pandemic. The results revealed living conditions and concerns about COVID-19 during the pandemic, concerns about student life during the COVID-19 pandemic, and specific concerns about the future and career paths. Furthermore, some of these items proved to affect students' subjective views of health. This is the first finding to identify factors related to anxiety and lifestyle related to subjective health views among university students at a comprehensive university in Japan during the COVID-19 pandemic.

The study revealed that the group with lower subjective views of health had lower satisfaction with sleep, diet, and university life, slept less, and did not exercise regularly. Previous studies have also reported that lifestyle changes due to COVID-19 decreased sleep quality and increased snacking and meal frequency (*10,11*). As for exercise habits, many studies have reported a decrease in physical activity due to lockdown (*12*) (*13*). It is likely that the respondents in this study were also affected by the government's declaration of a state of emergency and the switch to online lectures, which resulted in a significant change in their lifestyles. With regard to sleep, it is thought that disruption of sleep patterns due to decreased activity and an increase in the number of late nights may have contributed to the decline in sleep satisfaction (*14*). As for diet, it is possible that the restriction of activities to prevent the spread of infection may have caused changes in diet (*15*). Intriguingly, our result showed that high percentage of the students with high subjective health views proved to substitute meals with snacks. It is not clear whether

the cereal bars, marketed as nutritional supplements, are included in the "snacks" in their answers, though it is reported that increased consumption of cereals with high fruit and protein content during the COVID-19 pandemic may reflect increased health consciousness (*16,17*). Further detailed research is needed in this regard.

Next, we examined anxiety about the COVID-19 pandemic and found that those with low subjective health perceptions were more anxious about COVID-19 but less likely to practice infection-prevention behaviors, such as hand washing and gargling. As for the grade, the fourth-year students were more anxious and the first-year students were less anxious compared to the students of other grades. One possible reason is that young people at that time may have had a false perception that infection does not cause serious illness (*18*). Although this study period was between the third and fourth waves of COVID-19 epidemic in Japan, most of the severe cases were reported among elderly people or people with underlying diseases (*19,20*). Therefore, it is possible that the first-year students did not consider COVID-19 as something that would affect them. It is also possible that the general public understood the necessity of wearing masks and disinfecting their hands, and that the mucous membranes of the nose and mouth could be a route of infection but may not have been fully aware of the risk of infection through irregular hand washing and *via* eyelid mucosa. However, the effectiveness of regular handwashing, eye protection, and physical distance have been shown to decrease infection rates (*2,21*). It has also been reported that poor sleep quality can affect the immune system (*22*). The results of anxiety in students' life depended on the situation of each grade. On the one hand, the first-year students were anxious about the implementation of lectures, obtaining credits, and maintaining and building friendships on campus, similar to the results of the previous study (*23*). On the other hand, the third-year students were anxious about job seeking, internships, and whether they could find a job. At the time of the survey, one year had passed since the onset of the pandemic, and various measures, including remote lectures, were likely implemented so that students could continue their academic activities. However, a certain number of students felt uneasiness about each item, which could be a result of the lack of visibility in the future due to the COVID-19 pandemic. However, the high level of stress associated with social and physical environmental changes and adaptation to a new community is part of university life, in general (*24*). With regard to university students' mental health, the estimated prevalence of mood or anxiety disorders is reported to be 30.6% and is a predictor of depression if left unresolved (*25*). Depression may lead to various health behaviors such as unhealthy eating habits and poor sleep quality (*26*) and may lead to a vicious cycle.

Finally, the results presented here revealed that not only daily living conditions such as diet, sleep, and exercise habits but also anxiety about possible infection even from minimal outings necessary for daily life influenced subjective views of health. Noteworthily, dietary changes due to the COVID-19 pandemic may increase the risk of infection due to reduced immunocompetence caused by nutritional deficiencies (*27*). Previous studies have also indicated that the COVID-19 pandemic is associated with lower nutrient intake and health-related anxiety (*4,28*). It would be necessary to support university students to acquire relevant knowledge such as infection prevention measures to relieve their anxiety about COVID-19 during the pandemic or in the post-COVID-19 era. Furthermore, universities should create a system to support undergraduate and postgraduate students in all aspects of their lives so that they can lead healthy student lives.

Despite the scope covered in this study, there are still some limitations. First, this is a cross-sectional study conducted between the third to fourth major waves of the COVID-19 epidemic in Japan, and there was no comparison of the living conditions of students before and during the COVID-19 pandemic; therefore, it is unclear to what extent living conditions actually changed. Regarding the infection control measures implemented and factors of anxiety, the results may differ depending on the future status of the pandemic and measures such as vaccines and treatments utilized. Therefore, it is necessary to conduct a further survey on the living conditions of university students and the implementation of infection control measures according to the changing situations and concerns about university life in the future. However, the strength of this study is the first to examine whether the living conditions and anxiety of university students attending a comprehensive university in Japan during a pandemic affected their subjective views of health. The findings of this study may be utilized in future interventions to assist university students to lead healthy student lives.

5. Conclusions

This study, conducted during the COVID-19 pandemic, revealed that approximately half of the university students subjectively perceived themselves as unhealthy and had concerns about COVID-19, student life, their employment, and their future. Furthermore, satisfaction with living conditions and anxiety about student life and the future were found to influence the students' subjective health views. This is the first finding of its kind in Japan. The current COVID-19 pandemic provides an important opportunity to consider individualized needs-based supports to help students live a healthy life in the university. Even after the pandemic, it is important to understand the details of the concerns of undergraduate and postgraduate students and to consider support that meets their needs.

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*Address correspondence to:
Morihiro Ito, Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan.
E-mail: m-ito@isc.chubu.ac.jp

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Brief Report

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Examination of the utility of the COVID-19 detection kit, TRC Ready® SARS-CoV-2 i for nasopharyngeal swabs

Satoru Ishii[1,*], Moto Kimura[2], Tohru Miyoshi-Akiyama[3], Ataru Moriya[4], Masami Kurokawa[4], Erina Isaka[4], Junko Terada-Hirashima[1], Jin Takasaki[1], Shinyu Izumi[1], Masayuki Hojo[1], Haruhito Sugiyama[1]

[1] Department of Respiratory Medicine, National Center for Global Health and Medicine, Tokyo, Japan;
[2] Center for Clinical Sciences, National Center for Global Health and Medicine, Tokyo, Japan;
[3] Research Institute, National Center for Global Health and Medicine, Tokyo, Japan;
[4] Laboratory Testing Department, National Center for Global Health and Medicine, Tokyo, Japan.

SUMMARY The reverse transcription polymerase chain reaction (RT-PCR) offers high sensitivity, but has some drawbacks, such as the time required for the RNA extraction. Transcription reverse-transcription concerted reaction (TRC) Ready® SARS-CoV-2 i is easy to use and can be performed in about 40 minutes. TRC Ready® SARS-CoV-2 i and real-time one-step RT-PCR using the TaqMan probe tests of cryopreserved nasopharyngeal swab samples from patients diagnosed with COVID-19 were compared. The primary objective was to examine the positive and negative concordance rates. A total of 69 samples cryopreserved at -80° C were examined. Of the 37 frozen samples that were expected to be RT-PCR positive, 35 were positive by the RT-PCR method. TRC Ready® SARS-CoV-2 i detected 33 positive cases and 2 negative cases. One frozen sample that was expected to be RT-PCR positive was negative on both TRC Ready® SARS-CoV-2 i and RT-PCR. In addition, one frozen sample that was expected to be RT-PCR positive was positive by the RT-PCR method and negative by TRC Ready® SARS-CoV-2 i. Of the 32 frozen samples that were expected to be RT-PCR negative, both the RT-PCR method and TRC Ready® SARS-CoV-2 i yielded negative results for all 32 samples. Compared with RT-PCR, TRC Ready® SARS-CoV-2 i had a positive concordance rate of 94.3% and a negative concordance rate of 97.1%. TRC Ready® SARS-CoV-2 i can be utilized in a wide range of medical sites such as clinics and community hospitals due to its ease of operability, and is expected to be useful in infection control.

Keywords SARS-CoV-2, RT-PCR, TRC, Ct

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported in Wuhan City in the Hubei province of China in December 2019 and then spread globally. All genetic sequences of the virus were released on January 12, 2020 (*1*). The World Health Organization (WHO) declared it a pandemic on March 11, 2020 (*2*). The infection spread to Japan in February 2020, and a state of emergency was declared on April 7, 2020.

Data that can be used for COVID-19 diagnosis, such as severity markers (*3*) and diagnostic imaging (*4*) are increasing, but definitive diagnosis can be made by detecting pathogens, pathogen genes or pathogen antigens (*5*). Currently, COVID-19 testing uses a method of amplifying and detecting RNA sequences specific to the novel coronavirus by the reverse transcription polymerase chain reaction (RT-PCR) method. Although RT-PCR is characterized by high sensitivity, there are drawbacks, such as the requirement for manual performance of RNA extraction and the time-consuming nature of the measurements. Consequently, ways to reduce the burden on the laboratory technician and obtain results efficiently are needed.

The transcription reverse-transcription concerted reaction (TRC) method, which is a nucleic acid amplification test that amplifies and detects RNA at a constant temperature, can also be used (*6*). Using this method, the TRC Ready® system (Tosoh Bioscience, Tokyo, Japan) automates each process of the nucleic acid amplification test and has individually packaged reagents. The TRC Ready® system is also useful for detecting *Norovirus*, *Mycobacterium tuberculosis*

complex, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycoplasma pneumoniae*, *Neisseria gonorrhoeae* and *Chlamydia trachomatis*.

In the present study, the results from TRC Ready® SARS-CoV-2 i and of RT-PCR with cryopreserved nasopharyngeal swab samples of patients diagnosed with COVID-19 at our hospital were compared.

2. Materials and Methods

2.1. Study design and methods

The nasopharynxes of COVID-19 patients diagnosed at the National Center for Global Health and Medicine from October 2, 2020 to January 15, 2021 were wiped with FLOQSwabs (COPAN, Murrieta, CA, USA) and a general-purpose transport medium UTM (COPAN) was used. This study included 69 specimens that had been cryopreserved at -80°C.

The samples used in this study came from patients who participated in the prospective observational study of novel coronavirus infections (NCGM-G-003472-03) or the Filmarray Respiratory Panel 2.1 collection and storage of surplus specimens after examination (NCGM-G-004174-00) at the National Center for Global Health and Medicine. The subjects were those who consented to the secondary use of the stored specimens among those who participated in the study of collection and storage of surplus specimens after the examination (NCGM-G-004174-00). A retrospective study was conducted with the approval of the Ethics Committee (NCGM-G-004073-01).

2.2. Primary and secondary objectives

The primary objective was to examine the positive and negative concordance rates for TRC Ready® SARS-CoV-2 i compared with RT-PCR. The secondary objective was to compare the viral load of cryopreserved positive specimens with the cycle threshold (Ct). To confirm whether SARS-CoV-2 genes were detected in the samples for which TRC Ready® SARS-CoV-2 i and RT-PCR were not in concordance, residual liquid from nucleic acid purification was measured with real-time PCR, and sequence analysis was performed using an analysis primer for the obtained PCR products.

2.3. Inclusion criteria

Inclusion criteria for the study were age of at least 20 years, those who had a stored nasopharyngeal swab sample, and participants who consented to the "Prospective observational study of coronavirus infectious disease" (NCGM-G-003472-03).

2.4. Real-time one-step RT-PCR method using the TaqMan probe

The real-time one-step RT-PCR method using the TaqMan probe (QIAGEN, Tokyo, Japan) is the most commonly used method in Japan, and it involves a real-time reverse transcription-PCR method with hydrolysis probe using complementary strand DNA reverse transcribed from viral RNA as a template.

The QIAamp Viral RNA Mini Kit (QIAGEN) was used to isolate samples from viral RNA. RT-qPCR was performed using StepOne plus (Applied Biosystems, Tokyo Japan) according to the protocol of the National Institute of Infectious Disease, Japan (*7*).

The hydrolysis probe method uses a reporter dye (R) as a fluorescent dye at the 5' end and a 20-30 mer oligonucleotide labeled with a quencher (Q) at the 3' end. When the probe is intact, the reporter dye is quenched by the quencher and fluorescence resonance energy transfer, thus suppressing fluorescence. The probe then hybridizes to the virally derived cDNA that was reverse transcribed from viral RNA.

Subsequently, as the extension reaction by the primer proceeds, the probe is hydrolyzed by TaqDNA polymerase, so that the reporter dye is released from the probe and fluoresces. When probe hybridization and the PCR reaction are repeated for 40 cycles, the amount of free reporter dye increases in proportion to the amount of PCR product, so that the growth curve of the target gene sequence can be created in real time by monitoring fluorescence intensity. Those whose amplification curve is confirmed within 40 cycles are considered positive. Detection of SARS-CoV-2 genes from specimens and calculation of copy numbers were based on the real-time one-step RT-PCR method using the protocol of the National Institute Diseases on the N2 set.

2.5. TRC Ready® SARS-CoV-2 i

The TRC Ready® SARS-CoV-2 i is a nucleic acid amplification test based on the TRC method, which combines a DNA probe labeled with an intercalator fluorescent dye and a constant temperature RNA amplification method to amplify and detect RNA in one step (*6*). When the SARS-CoV-2 RNA promoter primer and SARS-CoV-2 RNA antisense primer included in this kit bind to SARS-CoV-2 RNA, the enzymes (reverse transcriptase, RNA polymerase) use the substrates (dNTP, NTP, ITP) used for transcription, reverse transcription, *etc*., and RNA is amplified and synthesized.

In parallel with this RNA amplification reaction, the fluorescently labeled SARS-CoV-2 RNA detection probe binds to the amplified and synthesized RNA, and the fluorescence intensity increases, so the fluorescence intensity in the reaction solution is measured over time. Thus, SARS-CoV-2 RNA in the sample is detected. TRCReady® SARS-CoV-2 i was used for detection. The TRCR® Purification Kit and TRCR® P-ASSIST were used for purification. The assay was performed on the Automated TRCR Real-time Monitor TRCReady®-80.

TRCReady® SARS-CoV-2 i cut-off value is positive when the fluorescence ratio exceeds 1.3 within 20 minutes from the start of the reaction.

2.6. Statistical analyses

SPSS version 24 (IBM Corporation, Armonk, NY, USA) was used for all statistical analyses. Spearman's rank correlation coefficient was used for the significance test comparing the two groups, with significance set at $p < 0.05$.

3. Results and Discussion

A total of 69 samples that were cryopreserved at -80 °C were examined. The median time from freezing the sample at -80°C to testing was 288 days (range 263-349). Of these samples, 37 were expected to be RT-PCR positive based on the RT-PCR test results at the time of collection, and 32 were expected to be negative (Figure 1). Of the 37 frozen samples that were expected to be RT-PCR positive, 35 were positive by the RT-PCR method. Using TRC Ready® SARS-CoV-2 i, there were 33 positive cases and 2 negative cases.

One frozen sample that was expected to be RT-PCR positive was negative on both TRC Ready® SARS-CoV-2 i and RT-PCR. In addition, one frozen sample that was expected to be RT-PCR positive was positive by the RT-PCR method and negative by TRC Ready® SARS-CoV-2 i.

For the 32 frozen samples that were expected to be RT-PCR negative, both the RT-PCR method and TRC Ready® SARS-CoV-2 i yielded negative results for all 32 samples (Table 1).

The positive concordance rate was 94.3% and the negative concordance rate was 97.1%. In the 35 patients who were positive by RT-PCR, median SARS-CoV-2 RNA viral load was 12,221 copies/mL (range 2-2,009,922), and median cycle threshold (Ct) was 27.3 (range 15.7-39.7). Examining the correlation between SARS-CoV-2 RNA viral load and Ct value, Spearman's rank correlation coefficient (r) was -0.591 and showed significance ($p = 0.0001$).

Ct values were high for the two samples that were positive by the RT-PCR method but negative by TRC Ready® SARS-CoV-2 i at 36.1 and 39.5 (Figure 2). Real-time PCR was performed for the nucleic acid purification products of the discordant samples. In a sequence analysis using analysis primers for the obtained PCR products, SARS-CoV-2 sequences were confirmed from all samples.

From the examination results, TRC Ready® SARS-CoV-2 i had a positive concordance rate of 94.3% and a negative concordance rate of 97.1% compared with the real-time one-step RT-PCR method using the TaqMan probe. The RT-PCR method offers high sensitivity and specificity and is widely used in Japan, but it is time-consuming and requires specific skills to perform

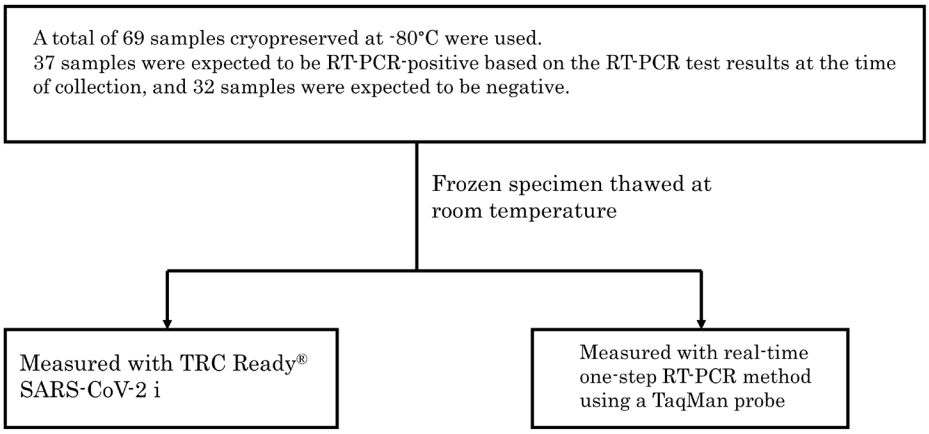

Figure 1. Study flowchart. A total of 69 nasopharyngeal swab samples that were cryopreserved at -80°C were examined. Of these 37 samples were expected to be RT-PCR positive based on RT-PCR test results at the time of collection and 32 samples were expected to be negative. Frozen samples were thawed to room temperature, then measured with the TRC Ready® SARS-CoV-2 i and real-time one-step RT-PCR method using a TaqMan probe.

Table 1. Comparison of results from TRC Ready® SARS-CoV-2 i and real-time one-step RT-PCR using the TaqMan probe for cryopreserved nasopharyngeal swab samples of patients diagnosed with COVID-19

		TRC Ready® SARS-CoV-2 i		Total
		Positive	Negative	
Real-time one-step RT-PCR method using the TaqMan probe	Positive	33	2	35
	Negative	1	33	34
Total		34	35	69

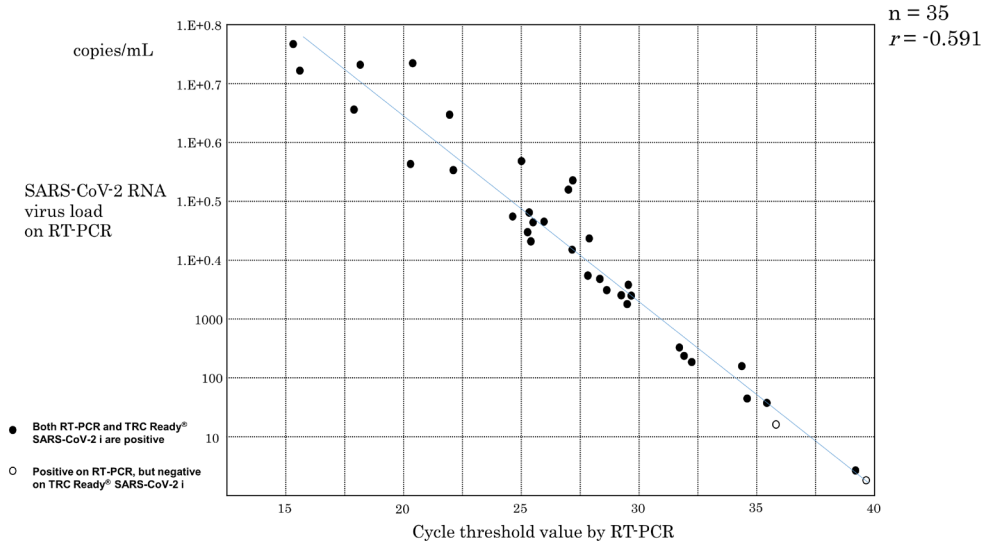

Figure 2. Correlation between SARS-CoV-2 RNA viral load and cycle threshold value in 35 RT-PCR positive patients. Examining the correlation between SARS-CoV-2 RNA viral load and Ct value, Spearman's rank correlation coefficient (r) was -0.591 (p = 0.0001). Ct values were high for the two samples that were positive by the RT-PCR method but negative by TRC Ready® SARS-CoV-2 i at 36.1 and 39.5.

appropriately.

On the other hand, TRC Ready® SARS-CoV-2 i provides the rapidity that is a feature of the nucleic acid amplification test method, and can be performed in about 40 minutes. In addition, six samples can be measured at the same time simultaneously, and the procedures are not difficult. The reaction reagents and starting solution of this kit are individually encapsulated for each measurement, so there is no need to prepare reagents at the time of measurement. Furthermore, since the reaction is completed in one closed tube, there is almost no risk of contamination. In addition, it is possible to perform measurement operations automatically using dedicated equipment.

With this kit, reaction inhibition originating in a sample can be detected by an internal control in the reagent, and false-negative results can be prevented. Real-time PCR was performed for the nucleic acid purification products of discordant samples, and in the sequence analysis, SARS-CoV-2 sequences were confirmed from all samples. Since there were no mutations in the regions amplified by RT-PCR or TRC Ready® SARS-CoV-2 i, the discordance is thought to have been caused by variation in measurements due to viral loads near the lower detection limit. Sethuraman *et al.* reported that high Ct levels make it difficult to isolate the virus responsible for an infectious disease from the sample, even if the genetic test is positive (*8*).

The TRC Ready® system is also useful for detecting *Norovirus*, *Mycobacterium tuberculosis* complex, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycoplasma pneumoniae*, *Neisseria gonorrhoeae* and *Chlamydia trachomatis* with a change of the reagents (*9,10*). Systems like TRC Ready®, which are easy to use and that can be used to detect various bacteria, can increase testing frequency and efficiency, thereby aiding in infection control in community hospitals.

4. Limitations

This was a retrospective study. Moreover, the number of positive and negative samples did not match because the samples were cryopreserved and usable.

5. Conclusion

Compared with the RT-PCR method, TRC Ready® SARS-CoV-2 i had a positive concordance rate of 94.3% and a negative concordance rate of 97.1%. TRC Ready® SARS-CoV-2 i can be used in a wide range of medical sites such as clinics and community hospitals due to its simple operability, and it is expected to be useful in infection control.

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**Address correspondence to:*
Satoru Ishii, Department of Respiratory Medicine, National Center for Global Health and Medicine, 1-21-1 Toyama Shinjuku-ku, Tokyo 162-8655, Japan.
E-mail: satishii@hosp.ncgm.go.jp

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Brief Report

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Histopathological analysis revealed that *Mycobacterium abscessus* proliferates in the fat bodies of silkworms

Yasuhiko Matsumoto[1,*], Hanako Fukano[2], Harutaka Katano[3], Yoshihiko Hoshino[2,*], Takashi Sugita[1]

[1] Department of Microbiology, Meiji Pharmaceutical University, Tokyo, Japan;
[2] Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan;
[3] Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo, Japan.

SUMMARY *Mycobacterium abscessus* causes chronic skin infections, lung diseases, and systemic or disseminated infections. Although a silkworm infection model with *M. abscessus* has been established, pathological analysis of the infected silkworms has not been performed. In this study, we performed hematoxylin-eosin and Ziehl-Neelsen staining of silkworms infected with *M. abscessus*. Four days after infection with *M. abscessus*, *M. abscessus* accumulation was observed in the fat bodies of silkworms. The number of viable *M. abscessus* cells in the fat bodies of the infected silkworms increased over time. These results suggest that *M. abscessus* proliferates in the fat bodies of the infected silkworms.

Keywords *Mycobacterium abscessus*, silkworm, fat body, infection

1. Introduction

Mycobacterium abscessus, is a group of rapidly growing non-tuberculous mycobacteria, causes chronic skin infections, lung diseases, and systemic or disseminated infections in immunocompromised patients (*1-4*). *M. abscessus* infections are associate with a greater lung function loss compared with cystic fibrosis caused by typical airway pathogens (*5*). High levels of antibiotic resistance in *M. abscessus* contribute to poor outcomes by limiting the treatment options (*6*). Several mouse *M. abscessus* infection models have been used to evaluate the efficacy of antibacterial drugs (*7*,*8*). These mouse infection models require several weeks to assess the mortality of infected mice. Moreover, long-term infection experiments using mice are difficult to conduct owing to animal welfare issues. The 3Rs, replacement, refinement, and reduction, are important animal welfare principles for experiments using mammals (*9*). To overcome these problems, a silkworm infection model with *M. abscessus* was established (*10*,*11*).

Silkworms are invertebrate animals that are beneficial for use in experiments to reveal host-pathogen interactions and identify candidates for antimicrobial drugs (*12-16*). Fewer ethical issues are associated with the use of a large number of silkworms for experimentation than with mammals (*17*). By exploiting this advantage of silkworms for infectious disease research, silkworm infection models are used as initial screening systems to identify virulence-related genes in pathogenic microorganisms (*18-21*). A silkworm infection model was established to evaluate anti-mycobacterial compounds using a type strain (*10*). Moreover, the virulence of *M. abscessus* clinical isolates was quantitatively evaluated by calculating the median lethal dose (LD_{50}), which is the dose of a pathogen required to kill half of the animals in a group, in a silkworm *M. abscessus* infection model (*11*). However, histopathological analysis of silkworms infected with *M. abscessus* has not yet been performed.

In the present study, we observed the accumulation of *M. abscessus* in the fat bodies of infected silkworms using histopathological analysis. Moreover, the number of viable *M. abscessus* cells increased in the fat bodies of infected silkworms. These findings suggest that the growth of *M. abscessus* in the fat bodies of infected silkworms might be a reason for the death of silkworms.

2. Materials and Methods

2.1. Reagents

Middlebrook 7H9 broth, Middlebrook 7H10 agar, and Middlebrook OADC enrichment were purchased from Becton, Dickinson, and Company (Sparks, MD, USA). Middlebrook 7H9 broth and Middlebrook 7H10 agar were supplemented with 10% Middlebrook OADC Enrichment. Hematoxylin-eosin (HE) was purchased

from Merck (Darmstadt, Germany). Ethanol, xylene, Ziehl carbol-fuchsine solution, and Loeffler's methylene blue solution were purchased from Muto Pure Chemicals Co. LTD (Tokyo, Japan).

2.2. Bacterial strain and culture condition

The *M. abscessus* subsp. *abscessus* ATCC19977 strain was used in this study. The *M. abscessus* ATCC19977 strain was grown on a Middlebrook 7H10 agar plate at 37°C. A single colony was then inoculated into 5 ml of Middlebrook 7H9 broth and incubated at 37°C for 3 days.

2.3. Infection experiments using silkworms

Silkworm infection experiments with *M. abscessus* were performed as previously described (*11*). Fifth-instar larvae were reared on an artificial diet (Silkmate 2S, Ehime-Sanshu Co., Ltd., Ehime, Japan) for 24 h. *M. abscessus* cells grown in Middlebrook 7H9 broth were collected by centrifugation and suspended in sterile saline. A 50-μL sample solution was administered to the silkworm hemolymph by injecting the silkworm dorsally using a 1-ml tuberculin syringe (Terumo Medical Corporation, Tokyo, Japan). Silkworms were injected with *M. abscessus* cells (1.4×10^7 cells per larva), and were incubated at 37°C for 4 days.

2.4. Histopathological analysis

Silkworms infected with *M. abscessus* were placed in 4% formalin and incubated at room temperature for two weeks (Figure 1). Paraffin-embedded sections of formalin-fixed silkworms were prepared and placed on glass slides.

HE staining was performed according to the manufacturer instruction. After immersion in xylene for 10 min, the specimens were immersed twice in 100% ethanol, and then in 95% ethanol, 80% ethanol, and 70% ethanol. After rinsing with water, specimens were immersed in Carracci's hematoxylin solution for 5 min. The specimens were immersed in 70% ethanol containing 0.2% hydrochloric acid, rinsed with water, and soaked in warm water for 10 min. After immersion in an alcohol eosin staining solution for 3 min, the specimens were quickly passed through 100% ethanol. The specimens were immersed in xylene for 3 min and sealed.

Ziehl-Neelsen staining was also performed according to the manufacturer instruction. Silkworm sections on glass slides were deparaffinized, removed xylene, and rinsed with distilled water. After immersion in Ziehl carbol-fuchsine solution for 30 min, the sections were rinsed under running water. After immersion in ethanol containing 0.5% hydrochloric acid for 10 seconds, sections were rinsed under running water. After 2 seconds of immersion in Loeffler's methylene blue solution, sections were lightly rinsed with water. The sections were dehydrated in 100% ethanol, immersed in xylene for 3 min, and sealed. Silkworm sections were examined under an optical microscope (BX51; Olympus, Tokyo, Japan).

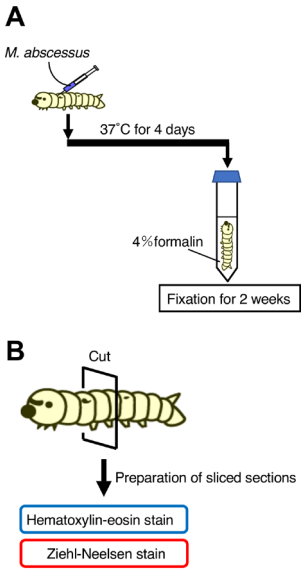

Figure 1. The experimental scheme of histopathological analysis of the silkworm infected with *M. abscessus* in this study. (**A**) Fixation of silkworms infected with *M. abscessus*. Silkworms were injected with saline or *M. abscessus* cells (1.4×10^7 cells per larva) and incubated at 37°C for 4 days. (**B**) Locations of sliced sections of silkworms.

2.5. Viable cell counts of *M. abscessus* in the silkworm fat body

Fat body isolation was performed as previously described (*22*). The silkworms were placed on ice for 15 min. Fat bodies were isolated from the dorsolateral region of each silkworm and rinsed with saline. The wet weights of the fat bodies were measured. The viable cell number of *M. abscessus* in the fat body of silkworms was measured according to a previous report (*23*). Silkworms were injected with *M. abscessus* cell suspension (7×10^6 cells in 50 μL) and incubated at 37°C. Fat bodies were harvested from silkworm larvae at 1, 2, 3, or 4 days post-infection. The fat body was homogenized in 1 mL of saline and the lysate was obtained. The lysate was 10^2 or 10^3-diluted with saline, and a 100 μL aliquot was spread on a Middlebrook 7H10 agar plate. The agar plate was incubated at 37°C for three days, and the colonies on the agar plates were counted. The viable cell number of *M. abscessus* per wet weight of fat body was determined.

2.6. Statistical analysis

The statistical significance of differences between viable cell counts of *M. abscessus* in silkworm groups was determined using Tukey's test. Statistical significance was set at $p < 0.05$.

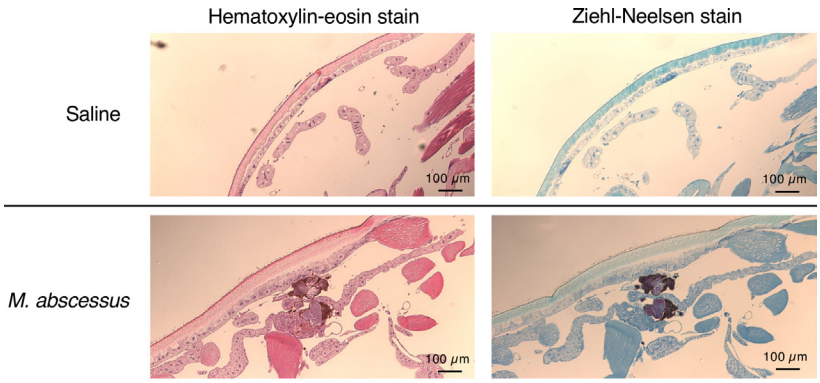

Figure 2. Histopathological analysis of sections from silkworms infected with or without *M. abscessus*. Silkworms were injected with saline or *M. abscessus* cells (1.4×10^7 cells per larva) and incubated at 37°C for 4 days. Silkworm sections were subjected to hematoxylin-eosin or Ziehl-Neelsen staining and observed under a microscope.

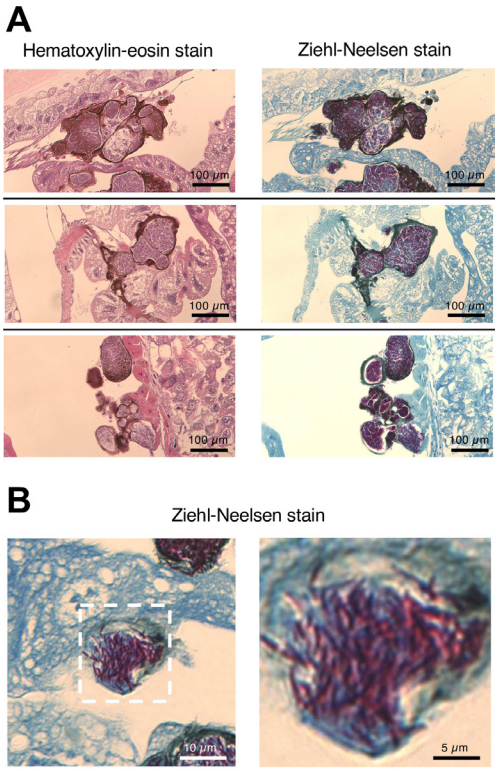

Figure 3. Accumulation of *M. abscessus* in the fat bodies of infected silkworm. (**A**, **B**) Silkworms were injected with *M. abscessus* cells (1.4×10^7 cells per larva) and incubated at 37°C for 4 days. Silkworm sections were subjected hematoxylin-eosin staining or Ziehl-Neelsen staining and observed under a microscope. (**A**) Three views of the fat bodies of the infected silkworm. (**B**) Red bacilli are observed.

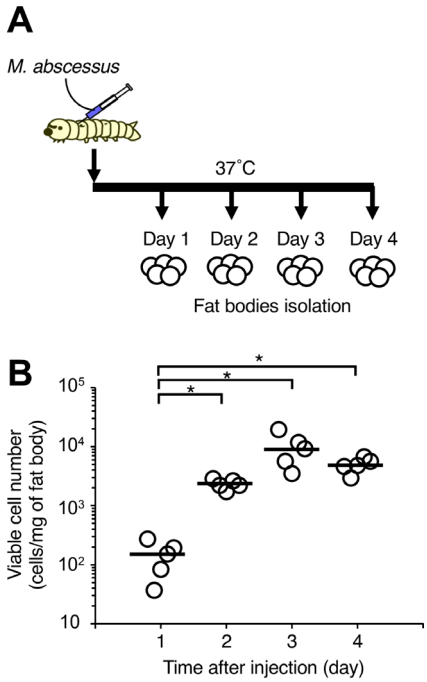

Figure 4. Increase in viable *M. abscessus* cells in the fat bodies of infected silkworms. (**A**) Experimental scheme. (**B**) Silkworms were injected with *M. abscessus* cells (7×10^6 cells in 50 μL) and were incubated at 37°C for 4 days. Silkworm fat bodies were isolated for four days after the injection of *M. abscessus*, and viable *M. abscessus* cells were counted. Five silkworms were used per group. The statistical significance of differences between viable cell counts of *M. abscessus* in silkworm groups was determined using Tukey's test. *: $p < 0.05$.

3. Results and Discussion

We performed histopathological analysis of the silkworms four days after infection with *M. abscessus*. *M. abscessus* cells (1.4×10^7 cells) were injected into silkworms, reared at 37°C for 4 days, and then immersed in 4% formalin solution for 2 weeks (Figure 1A). Silkworms were sliced into rings and subjected to hematoxylin-eosin and Ziehl-Neelsen staining (Figure 1B). In hematoxylin-eosin staining, a reddish brown area was observed in the fat bodies of the silkworms infected with *M. abscessus*, but not in those of the silkworms injected with saline (Figure 2). In the Ziehl-Neelsen staining, the area was stained dark red (Figure 2). Several areas stained dark red by Ziehl-Neelsen staining were observed in the fat bodies of silkworms infected with *M. abscessus* (Figure 3A). Red bacilli were observed in the fat bodies of silkworms infected with *M. abscessus* by Ziehl-Neelsen staining (Figure 3B). These results suggest that *M. abscessus* accumulates in the fat bodies of the silkworms.

Next, we examined whether *M. abscessus* grows in the fat bodies of the silkworms. The fat bodies of the silkworms were removed at 1, 2, 3, and 4 days after inoculation of silkworms with *M. abscessus*, and the number of viable *M. abscessus* cells in the fat bodies was determined (Figure 4A). The number of viable *M. abscessus* in the fat bodies of silkworms at 2, 3, and 4

days after inoculation with *M. abscessus* was higher than that at 1 day (Figure 4B). These results suggest that the fat body of the silkworm is the target of *M. abscessus* during infection.

In this study, *M. abscessus* accumulation was observed in the fat bodies of the silkworms. These foci differ from those of caseation necrosis, which are formed from aggregates of dead cells. A biofilm of *M. abscessus* complex in the pathologically thickened alveolar wall of the explanted lung of a patient with cystic fibrosis was observed (*24*). The foci of *M. abscessus* accumulation found in the infected silkworms were similar to those found in the lungs of a patient with cystic fibrosis. The establishment of a silkworm model of caseation necrosis caused by *M. abscessus* will be an important subject for future research.

In conclusion, we performed histopathological analysis of silkworms infected with *M. abscessus* and observed the accumulation of *M. abscessus* in their fat bodies. Furthermore, the number of viable *M. abscessus* cells in the fat bodies of silkworms infected with *M. abscessus* increased. These results suggest that *M. abscessus* grows in the fat bodies of the silkworms.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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**Address correspondence to:*
Yasuhiko Matsumoto, Department of Microbiology, Meiji Pharmaceutical University, 2-522-1, Noshio, Kiyose, Tokyo 204-8588, Japan.
E-mail: ymatsumoto@my-pharm.ac.jp

Yoshihiko Hoshino, Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, 189-0002, Japan.
E-mail: yhoshino@niid.go.jp

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Correspondence

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Does every *Staphylococcus aureus* infection require anti-MRSA drugs? Three case reports of a *Staphylococcus aureus* infection

Guangbin Chen[1], Hong-Zhou Lu[2,*]

[1] Department of Pharmacy, The Third People's Hospital of Shenzhen, Shenzhen, China;
[2] National Center for Infectious Diseases Research, The Third People's Hospital of Shenzhen, Shenzhen, China.

SUMMARY *Staphylococcus aureus* is a common clinical pathogen. Does every *S. aureus* infection require anti-MRSA drugs? Reported here are three cases of a community-acquired infection with S. aureus. The first case involveds a 45-year-old male who was admitted due to right ankle pain for 1 month; he was diagnosed with chronic suppurative osteomyelitis and an acute soft tissue infection of the ankle. S. aureus was cultured from the pus and was resistant to penicillin and sensitive to oxacillin and vancomycin. After receiving oxacillin, he was cured and discharged 45 days after admission. The second case involved a 44-year-old male who was admitted due to lumbar pain with right lower limb numbness for more than 1 month and fever for 1 day. *S. aureus* was cultured from blood specimens and was resistant to penicillin and sensitive to oxacillin and vancomycin. After receiving oxacillin, he as cured. The third case involved a 7-day-old newborn who was admitted due to skin jaundice for 6 days. *S. aureus* was cultured from skin secretions specimens and was resistant to penicillin and sensitive to oxacillin, erythromycin, and vancomycin. The newborn was treated with oxacillin for 4 days, and she was cured and discharged. Not all cases a suspected *S. aureus* infection require anti-MRSA drugs; instead, previous *S. aureus* susceptibility results in the area and hospital, as well as the patient's clinical profile, need to be taken into account.

Keywords *Staphylococcus aureus*, MRSA, antimicrobials, culture, adverse reactions

The abuse of antimicrobials is one of the reasons for increasingly serious antimicrobial resistance. *Staphylococcus aureus* is a common clinical pathogen. There are 3 forms of *S. aureus* that are sensitive to antibiotics. One is sensitive to penicillin, oxacillin, the first, second, third, and fourth generation of cephalosporins, and vancomycin, and this form does not produce B lactamases. The second form produces B lactamases and is commonly known as methicillin-sensitive *Staphylococcus aureus* (MSSA), which is resistant to penicillin but sensitive to oxacillin, all four generations of cephalosporins, and vancomycin. The third form is commonly known as methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant to penicillin, oxacillin, and many antibiotics including all four generations of cephalosporins. There are few antimicrobial agents available; only vancomycin, teicoplanin, linezolid and other drugs can be selected, and moxifloxacin and rifampin can be selected in some cases. Clinically, when some clinicians encounter suspected cases of an *S. aureus* infection, such as skin and soft tissue infections (SSTIs), they often choose anti-MRSA drugs such as vancomycin, linezolid, and teicoplanin for treatment empirically due to the concern that the case involves an MRSA infection before the bacterial culture results are received. Nevertheless, does every *S. aureus* infection require anti-MRSA drugs?

On one hand, the incidence of adverse reactions to drugs such as vancomycin, linezolid, and teicoplanin is relatively high and the symptoms of those adverse reactions are relatively serious. On the other hand, these drugs are expensive, causing a waste of medical resources, which does not conform to the principles of pharmacoeconomics. Therefore, understanding the drug resistance spectrum of pathogenic bacteria in this area, including the characteristics of the pathogen resistance spectrum in various hospital departments, is of great help to empirically selecting appropriate antibacterials before the results of a specimen bacterial culture are available. Below are three cases of a community-acquired infection with *S. aureus*, and the results of a culture of *S. aureus* in our hospital are analyzed.

The first case involved a 45-year-old male who was admitted to our hospital on September 7, 2021 due to right ankle pain for 1 month that had intensified over 4 days. The right ankle was swollen and painful after

acupuncture 4 days prior, and the skin temperature was high. He denied having a fever, cough, nausea, or vomiting. He also denied a history of chronic diseases such as hypertension and diabetes. A physical examination on admission revealed a normal body temperature of 36.6℃, a blood pressure of 123/80 mmHg, a normal sinus rhythm of 78 beats per minute, and a respiratory rate of 20 breaths per minute. The patient was conscious, cooperative during the physical examination, and coherent. A physical examination of the heart and lungs was normal. Swelling, tenderness, and an increased skin temperature were noted in the right ankle. Laboratory results revealed a C-reactive protein (CRP) level of 85.1 mg/L (normal range: 0 mg/L to 6 mg/L). The procalcitonin level of 0.12 ng/mL was within the normal range. The white cell count was 16.27 × 10^9/L and the neutrophil count was 13.15 × 10^9/L. Computed tomography (CT) on admission revealed osteomyelitis and a local abscess in the lower segment of the right tibia. The patient was diagnosed with chronic suppurative osteomyelitis and an acute soft tissue infection of the ankle.

Given that the most common pathogen causing chronic osteomyelitis and SSTIs is *S. aureus* and based on the drug resistance of *S. aureus* cultured from various specimens at our hospital, a consultation recommended oxacillin for empirical anti-infection therapy before obtaining the results of a bacterial culture and drug sensitivity test. The consultation was heeded and an oxacillin sodium injection was initiated starting on the day of admission. On the third day of hospitalization, vacuum sealing drainage (VSD) and tibial drainage were performed to treat osteomyelitis of the right tibia and a peripheral abscess. Four days later, *S. aureus* was cultured from the pus and was resistant to penicillin and sensitive to oxacillin and vancomycin. Oxacillin was continued (2.0 g, *i.v.*, q8h) for 6 weeks. Fourteen days after admission, secondary osteomyelitis of the right tibia was removed, and debridement and suturing were performed. Forty-five days after admission, the patient was cured and discharged.

The second case involved a 44-year-old male who was admitted to our hospital on February 9, 2019 due to lumbar pain with right lower limb numbness for more than 1 month and a fever for 1 day. He had a history of chronic hepatitis B with cirrhosis and diabetes. A physical examination on admission revealed a body temperature of 40.1℃, blood pressure of 122/71 mmHg, sinus tachycardia of 140 beats per minute, and a respiratory rate of 21 breaths per minute. Laboratory results revealed a CRP level of 92.25 mg/L. The procalcitonin level of 0.899 ng/mL was high (normal range: 0 to 0.5 ng/mL). The white cell count was 7.86 × 10^9/L, and the neutrophil count was 6.43 × 10^9/L. CT revealed bone destruction of the fourth lumbar vertebra with a soft tissue mass, and the possibility of tuberculosis with a paraspinal cold abscess was considered. After admission, the patient's condition was as follows: 1) The lumbar lesion was investigated for a potential tuberculous infection, 2) Type 2 diabetes mellitus, and 3) Chronic hepatitis B with cirrhosis. Since the pathogen was unknown, a tuberculous infection and brucellosis could not be ruled out, and levofloxacin and amikacin were empirically initiated starting on the day of admission. Ten days after admission, *S. aureus* was cultured from blood specimens and was resistant to penicillin and sensitive to oxacillin, levofloxacin, amikacin, and vancomycin. Levofloxacin was stopped and oxacillin was substituted. Lumbar debridement was performed. Fifty days after admission, the patient was cured and discharged.

The third case involved a 7-day-old newborn who was admitted to this hospital on September 22, 2020 due to skin jaundice for 6 days and a rash for 1 day. After admission, the patient's condition was as follows: 1) Neonatal impetigo and 2) Neonatal hyperbilirubinemia. The child's general condition was good. Given that the most common pathogen causing cutaneous impetigo is *S. aureus*, oxacillin was used empirically. Symptomatic treatment was also administered. *S. aureus* was cultured from skin secretions and was resistant to penicillin and sensitive to oxacillin, cefazolin, erythromycin, and vancomycin. The newborn was treated with oxacillin for 4 days, and she was cured and discharged.

All three of these cases have the following common characteristics: 1) *S. aureus* cultured from various specimens was resistant to penicillin but sensitive to oxacillin, various generations of cephalosporins, and vancomycin, 2) The patient's general condition was satisfactory and the clinical symptoms were not very serious, and 3) Oxacillin was efficacious.

A large-scale study of the HealthCore Integrated Research Database found that the incidence of SSTIs was approximately 48.46 cases/1,000 people per year (*1*). Another study found that the main pathogenic bacteria causing SSTIs were Gram-positive and Gram-negative bacteria, and *S. aureus* was the most common pathogenic bacterium detected in SSTIs (*2*).

S. aureus is a common opportunistic pathogen. It mainly colonizes the groin, perineum, nasal vestibular mucosa, and neonatal umbilical cord stump and can occasionally colonize the skin and oropharynx. It can produce a variety of hemolytic toxins, leukocidin, enterotoxin, and plasma coagulase. It mainly causes a variety of diseases, including suppurative infections and inflammation of the skin, mucosa, and deep tissue, postoperative infections at various sites, deep abscesses in various organs, bacteremia, toxic-shock syndrome, and a microflora disorder involving the whole body (*3*). Some cases of an *S. aureus* infection have been serious and even fatal. *S. aureus* is one of the common pathogens infecting communities and hospitals. The skin and mucosa are generally believed to be the main sites of *S. aureus* colonization (*4*). In patients with

skin diseases, *S. aureus* colonization is likely to cause a local or systemic infection due to the damaged skin protective barrier.

The drug resistance of community-acquired *S. aureus* infections differs from that of nosocomial *S. aureus* infections. MRSA is considered to be the most common pathogen causing nosocomial infections. MRSA is reported to account for 25.5% of community-associated *S. aureus* infections and 67.4% of nosocomial infections in China, South Korea, and Japan (*5*). The rate of MRSA detection in the community is relatively low. The average rate of MRSA detection in some hospitals in China from 2014-2018 was 31.7%, and the rate of MRSA detection decreased from 69.0% in 2005 to 35.3% in 2017 (*6*).

From January 1, 2017 to October 31, 2022, 1,004 specimens of *S. aureus* infections were cultured in our hospital, and 540 of those specimens (53.8%) did not produce B lactamases. Three hundred and two specimens were MSSA (including 92 cases of the MLSB phenotype), accounting for 30.1%; 162 were MRSA, accounting for 16.1%. Regrettably, distinguishing between community-acquired and nosocomial infections was not possible for those 1,004 specimens of *S. aureus*.

In recent years, there has been an increase in vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA), particularly in Asia (*7*). Nonetheless, the incidence of VISA and hVISA is low, i.e., less than 10%. MRSA resistant to vancomycin has not been reported in China. Therefore, vancomycin is still considered to be the preferred drug for an MRSA infection (*8*). Therefore, when clinicians encounter suspected cases of an *S. aureus* infection, vancomycin, linezolid, teicoplanin, and other anti-MRSA drugs are often used empirically before the results of a bacterial culture are available out of fear of MRSA and to ensure efficacy.

Compared to oxacillin or penicillin, vancomycin causes relatively more adverse reactions (*9*). The most serious adverse reactions can lead to death (*10*). Antimicrobial agents are the most common cause of drug reactions in the form of eosinophilia and systemic symptoms (DRESS), and vancomycin is responsible for about two-thirds of DRESS cases induced by antimicrobial agents (*11*). Vancomycin can cause renal impairment (*12,13*), as well as red man syndrome (*14*), thrombocytopenia (*15*), and severe exfoliative dermatitis (*16*). Other drugs commonly used to treat MRSA infection, such as teicoplanin and linezolid, cause relatively more adverse reactions and serious symptoms of adverse reactions.

Based on the information presented thus far, appropriate antibiotics can be selected empirically based on a comprehensive assessment of previous *S. aureus* drug sensitivity results in the region and the hospital, as well as the patient's clinical condition, in patients with suspected *S. aureus* infections before the culture results are received. This can avoid the waste of resources and avoid adverse reactions to drugs to the extent possible. If the incidence of MRSA in previous cultures of *S. aureus* in the local area and the hospital is low, *e.g.*, the rate of MRSA detection in our hospital is only 16.1%, and the patient's condition is not very serious, then initiation of oxacillin and other drugs such as cephalosporins can be considered. Antibiotics are then adjusted in accordance with the *S. aureus* drug sensitivity results and the patient's condition. Unless these patients have severe disease and are likely to have MRSA, then empirically selecting anti-MRSA drugs such as vancomycin, linezolid, and teicoplanin for all suspected cases of *S. aureus* infection is not necessary.

In conclusion, not all suspected cases of *S. aureus* infection require anti-MRSA drugs. Instead, previous *S. aureus* susceptibility results in the area and hospital, as well as the patient's clinical profile, need to be taken into account.

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**Address correspondence to:*
Hongzhou Lu, Department of Infectious Diseases, National Clinical Research Center for Infectious Diseases, the Third People's Hospital of Shenzhen, 29 Buji Bulan Road, Shenzhen, Guangdong Province 518112, China.
E-mail: luhongzhou@fudan.edu.cn

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Comment

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Suppressing host pyroptosis by a ubiquitin-activated phospholipid phosphatase of *Mycobacterium tuberculosis*

Qishun Feng, Guoliang Zhang, Hongzhou Lu*

National Clinical Research Center for Infectious Diseases, Guangdong Provincial Clinical Research Center for Tuberculosis, Shenzhen Third People's Hospital, Southern University of Science and Technology, Shenzhen, China.

SUMMARY Tuberculosis (TB) is a major public health problem that causes millions of deaths in humans around the world, and the bacterial pathogen *Mycobacterium tuberculosis* (Mtb) is responsible for this disease. Evidence suggested that the inflammasome-pyroptosis pathway is crucial for preventing Mtb infection. Uncertainty exists regarding whether and how these infections can bypass this immune system by Mtb. A recent *Science* article by Chai *et al*. (doi: 10.1126/science.abq0132) revealed a novel role by a eukaryotic-like effector called PtpB during Mtb infection. The PtpB functions as a phospholipid phosphatase suppressing gasdermin D (GSDMD) dependent pyroptosis. And notably, the phospholipid phosphatase activity of PtpB is dependent on binding with mono-ubiquitin (Ub) of the host.

Keywords *Mycobacterium tuberculosis*, phospholipid phosphatase, ubiquitination, pyroptosis

Mycobacterium tuberculosis (Mtb) is a fatal bacterial pathogen that causes tuberculosis (TB) in humans (*1*). It is estimated that one-third of the global population has been infected with Mtb (*2*). In 2021, a total of 1.6 million people died from TB worldwide (*3*). The inflammasome-pyroptosis pathway is critical for immune defense when the host encounters the invasion of pathogenic microorganisms like Mtb (*4,5*). However, it remains unclear whether and how these infections are able to bypass this immune system. Therefore, it is anticipated that understanding how the major effectors of Mtb and other pathogens manipulate the host inflammasome-pyroptosis pathway may lead to the identification of novel anti-TB targets and intervention techniques.

In a recent study, by using a recombinant system of absent in melanoma 2 (AIM2) and NOD-like receptor protein 3 (NLRP3) inflammasomes in HEK293T cells, Chai *et al*. carried out a comprehensive screening of Mtb-encoded eukaryotic-like secretory proteins. They discovered that the Mtb-secreted protein phosphatase PtpB which can inhibit host inflammasome-pyroptosis pathway. The authors present a model that PtpB dephosphorylates host plasma membrane components phosphoinositides phosphatidylinositol-4-monophosphate (PI4P) and phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2], thus disrupting N-terminal cleavage fragment of GSDMD (GSDMD-N) dependent inflammatory cytokine release and pyroptosis (Figure 1). And surprisingly, the phosphatase activity of PtpB is activated by binding ubiquitin (Ub) of the host (*6*).

The membrane of host could be a target on the battlefield of pathogen-host interactions. Other groups have reported that in *Legionella pneumophila*, effectors phosphatidylinositol (PI) 3-kinase MavQ and PI 3-phosphatase SidP dynamically remodel the membrane of the host (*7*). And in *Shigella flexneri*, effector IpgB1 binds to acidic phospholipids and regulates actin filament dynamics (*8*). However, whether Mtb could also invade the host by targeting its membrane, and whether there is a link among bacterial effector, host membrane and inflammasome-pyroptosis, if the answer is yes, the underlying mechanism had yet to be addressed. The first highlight in the work of Chai and colleagues is that they answer this question and present the evidence for how Mtb disrupts the membrane components PI4P and PI(4,5)P2, both of them are reported to be important for GSDMD-N mediated pyroptosis (*9,10*). The authors showed that expression of PtpB prominently reduced the membrane localization of GSDMD-N and deletion of *ptpB* of Mtb significantly increased the translocation of GSDMD-N to the plasma membrane of the host.

The second highlight in this work is that they elucidated the novel function mechanism of effector PtpB. Although there are several studies about PtpB before (*11-15*), as a phosphatase, *in vitro* conditions, the phosphatase activity of PtpB is not easily detectable (*16,17*), which implied the activity of PtpB might be regulated *in vivo*. Chai *et al*. first showed that PtpB dramatically decreased the amounts of PI4P and PI(4,5)P2 in the plasma membrane of the host. Furthermore, the

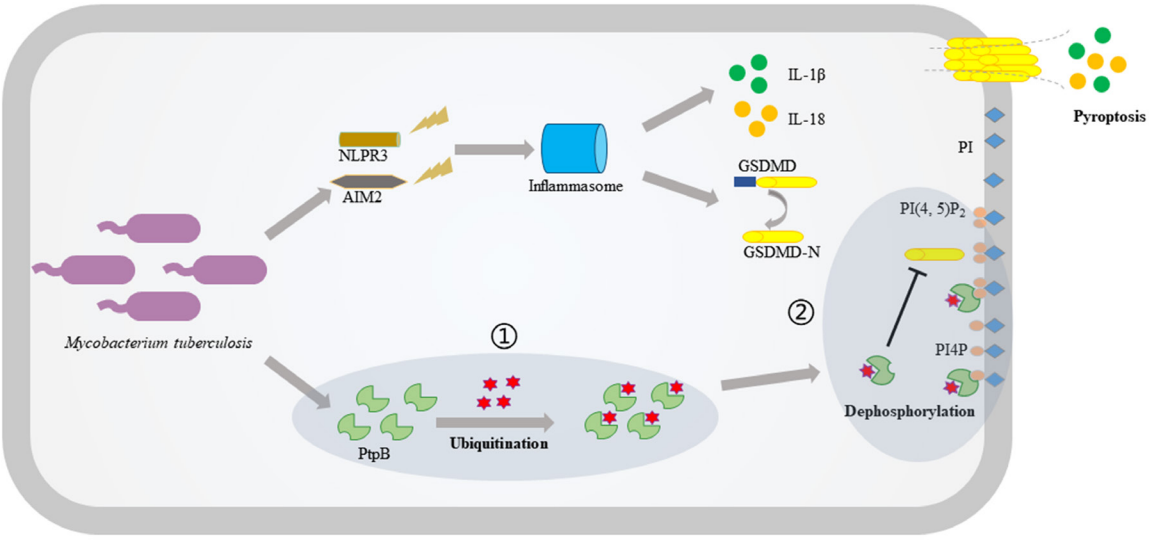

Figure 1. Schematic diagram of PtpB mediated GSMND-dependent pyroptosis during Mtb infection. Firstly, the PtpB secreted by Mtb exploits the Ub of the host to enhance its phospholipid phosphatase. Secondly, the activated PtpB dephosphorylates PI4P and PI(4,5)P2, which disrupts GSMND-N to localize the membrane to form a pore. Thereafter, the release of cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) from the pore and as well as the pyroptosis are inhibited. The numbers with circles show the two key processes that PtpB is involved in, *i.e.*, ubiquitination and dephosphorylation.

author's structural analysis showed that PtpB possesses an ubiquitin interacting motif (UIM)-like region, next they proved that only mono-Ub, rather than poly-Ub can bind to PtpB and then activates its activity. It is assumed that upon binding with Ub, PtpB would undergo a conformational change to expose its active site for substrate binding. And interestingly, it seems that the activity of UIM-like domain containing enzyme is stringently regulated by Ub, the author's previous work has shown that another secreted tyrosine phosphatase PtpA of Mtb could also suppress innate immunity by binding ubiquitin of the host *via* its UIM-like domain (*18*). Nevertheless, their targets are quietly different, which implied the versatile roles of the homologous phosphatases. Thus, this work presents us with a novel role of Ub activated PtpB by targeting the host's membrane to inhibit pyroptosis.

However, there are still a few unanswered questions. Firstly, it remains unknown why only mono-Ub rather than poly-Ub activates PtpB, and how mono-Ub is added to PtpB, whether this process is achieved by the canonical ubiquitination pathway of the host (*19*), or whether there is a novel enzyme that can ubiquitinate PtpB with mono-Ub, finding the enzyme(s), and the underlying mechanism of the particular ubiquitination process awaits further study in details. Secondly, the authors showed that except for IL-1β and IL-18, PtpB also suppressed macrophages' production of tumor necrosis factor (TNF-α) and IL-6, in a way that was independent of the GSDMD, which is consistent with previous studies that PtpB also inhibits non-inflammasome immune pathways (*14,15*). The increased intracellular survival of Mtb by PtpB could be a combinational outcome of the inflammasome and non-inflammasome immune pathways. Along those lines, it is possible that there is a balance between these two different pathways, and it also raises the question that what is regulator(s) that influence PtpB to function, by inflammasome or non-inflammasome immune pathways? Currently, at least we know the Ub is a determinant of PtpB-mediated inflammasome immune pathways. Whether other regulator(s) exist, and how are they regulated remains to be explored.

From a perspective of development of anti-TB therapeutics, the finding of the novel role of PtpB is valuable and it shows great potential. Firstly, the authors showed that GSDMD could confer robust host protective immune responses against infection. And when Mtb lacks functional PtpB, it cannot counteract host GSDMD-mediated immunity. It is possible that PtpB could be a desirable target for enhancing the immunological efficacy of Bacille Calmette-Guérin (BCG), since it possesses an identical PtpB-encoding gene with Mtb. Secondly, PtpB might be a promising anti-TB target for drug design, because there is no homologous protein of PtpB in humans, which implied excellent selectivity. Based on the two key processes PtpB is involved in, i.e., ubiquitination and dephosphorylation, anti-TB strategies could be developed. Small molecule compounds that block the novel found ubiquitin binding site (Ala240 -Ala242) or key enzyme active site (Cys160) of PtpB might abrogate PtpB's immune system suppression. Thirdly, PtpB could also be an intriguing target by using newly-developed proteolysis targeting chimeras (PROTACs) technology (*20*). Degrades PtpB through the ubiquitin-proteasome system could abolish PtpB mediated pyroptosis inhibition.

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**Address correspondence to:*
Hongzhou Lu, National Clinical Research Center for Infectious Disease, The Second Affiliated Hospital, Southern University of Science and Technology, 29 Bulan Road, Long Gang District, Shenzhen 518112, Guangdong, China.
E-mail: luhongzhou@fudan.edu.cn

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Editorial and Head Office
Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan.
E-mail: office@ddtjournal.com