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Brief Report

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CCL5/RANTES is important for inducing osteogenesis of human mesenchymal stem cells and is regulated by dexamethasone

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Summary **In this study, we examine the effect of chemokine (C-C motif) ligand 5 (CCL5)/Regulated on Activation Normal T cell Expressed and Secreted (RANTES), a pro-inflammatory cytokine on osteogenic differentiation of human mesenchymal stem cells (hMSCs). We found CCL5 expression was increased during osteogenic differentiation of hMSCs and CCL5 expression is dependent on the presence of dexamethasone. Knocking down endogenous CCL5 expression blocked osteogenesis, as revealed by decreasing alkaline phosphatase (ALP) activity and a reduction in the expression levels of ALP, bone sialoprotein (BSP), and osteopontin (OPN). Of note, the overexpression of CCL5 was sufficient to increase ALP expression and activity. Moreover, the down-regulation of chemokine (C-C motif) receptor 1 (CCR1), one of the CCL5 receptors, significantly decreased the osteogenesis of hMSCs. Interestingly, the down-regulation of CCR1, but not CCL5, was sufficient to affect the cell numbers during the process of osteogenesis. Our findings reveal that both CCL5 and CCR1 are required for osteogenesis of human MSCs, CCL5 is sufficient for the osteogenesis, and provide a novel link between dexamethasone and CCL5 in human osteogenesis.**

Keywords: CCL5, osteogenesis, mesenchymal stem cell, dexamethasone, CCR1

1. Introduction

Bone is a tissue with continuous turnover and is maintained and manipulated by two opposite processes. One is bone formation by osteoblasts originating from mesenchymal stem cells (MSCs), and the other is bone resorption by osteoclasts derived from macrophages. When the balance between bone formation and resorption is disrupted, bone diseases such as osteoporosis occur. Some signaling pathways related to inflammation have been suggested to be involved in osteoblast and osteoclast differentiation or formation (*1*). For example, chemokine (C-C motif) receptor 4 (CXCR4) deficiency impairs osteogenic differentiation of MSCs (*2*), while interleukin-1 (IL-1) promotes osteoclast formation. To explore the interplay between inflammation signaling and osteogenesis, we examined the roles of chemokine (C-C motif) ligand 5 (CCL5) function in bone formation.

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CCL5, also called Regulated on Activation Normal T cell Expressed and Secreted (RANTES), is a pro-inflammatory chemokine. CCL5 was reported to play important roles in chemo-attraction (*3*), recruitment of leukocytes to the sites of injury and inflammation, and carcinogenesis (*4-5*). In CCL5 deficient mice, the effect of loss of CCL5 is dependent upon different age. Only in 6-month-old mice, but not in young mice or 12-month-old mice, bone volume and bone formation rate (BFR) were observed to be decreased (*6*). In a mouse model, CCL5 had been shown to promote chemotaxis and survival of osteoblasts (*7*). The function of CCL5 in osteogenesis, particularly the effects of CCL5 in the process of MSC differentiate into osteoblast, has not been determined in human cells.

CCL5 can mediate signals through three receptors: chemokine (C-C motif) 1 (CCR1), chemokine (C-C motif) receptor 3 (CCR3), and chemokine (C-C motif) receptor 5 (CCR5) (*8*). The profile of chemokine receptors was analyzed by flow cytometry of human MSCs, and the results revealed that at least 70% of MSCs express CCR1, while none express CCR3 or CCR5 (*9*). Moreover, modulating the receptor activator of nuclear factor-κB ligand (RANKL)/RANK mediated interaction and osteoclast/osteoblast function in a mouse model (*10*), and osteopenia occur within CCR1-deficienct mice where they have fewer and thinner trabecular bones.

The regulatory mechanisms of CCL5 are unknown in osteogenesis of human MSCs. Dexamethasone, a glucocorticoid hormone, is well known for its function to modulate the osteoblast differentiation of human MSCs and augmenting alkaline phosphatase (ALP) activity. Low dosage of dexamethasone functions induced osteogenesis as inducer of osteogenesis, but high dosage of dexamethasone suppress osteogenesis. The relation between dexamethasone and CCL5 in the progress from hMSCs into osteo-committed cells has not been investigated.

In this study, we demonstrated that CCL5 was essential and sufficient for osteogenesis and also found the vital roles of CCR1 in the osteogenesis of human MSCs. Interestingly, we also found that CCL5 expression levels increased upon osteogenesis and were regulated by dexamethasone.

2. Materials and Methods

2.1. *Cell culture*

Human primary bone marrow (hMSCs) were obtained from Lonza (Basel, Switzerland) and cultured in MesenPRO RS media (Invitrogen, Carlsbad, CA, USA). For osteoblast differentiation, hMSCs were treated with low glucose DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 0.1 μM dexamethasone (Sigma, St. Louis, MO, USA), 10 mM β-glycerophosphate (Sigma), and 0.05 mM L-ascorbic acid phosphate (Sigma). The differentiation media were replaced twice weekly during the process of osteogenesis. Recombinant CCL5 (Peprotech, Rocky Hill, NJ, USA) was given to promote hMSC differentiation as indicated.

2.2. *Lentivirus production and infection*

Lentivirus production was performed as described previously (*11*). The shRNA (small hairpin RNA) lentiviruses against CCL5 (shCCL5-1: TRCN0000058005, 5'- GTATTTCTACACCAGTGGCAA -3'; shCCL5-2: TRCN0000371627, 5'-CCTGCTGCTTTGCCTACATTG -3') and CCR1 (shCCR1-1: TRCN0000008184, 5'-CCCTGGTAGAAAGAAGATGAA -3'; shCCR1-2: TRCN0000273656, 5'-ATTCTGCTAAGACGACCAAAT-3') were purchased from the National RNAi Core Facility (Taipei, Taiwan). Cells were infected with the shRNA lentiviruses of CCL5, CCR1, red fluorescence protein (RFP) in the presence of 8 μg/mL protamine sulfate, and the media were changed to differentiation media at 24 h post-infection.

2.3. *Alamar Blue assays and alkaline phosphatase activity assays*

For measuring the relative cell number, cells were cultured in the presence of 10% Alamar Blue reagent (Bio-Rad, Oxford, UK) for 1 h and the absorbance was measured at wavelength of 570 nm/600 nm. The relative cell numbers were calculated. Alkaline phosphatase activity assays were performed as previously described (*11*). The absorbance was measured at OD 405 nm, and the fold change of ALP activity was normalized to corresponding cell numbers measured in the Alamar Blue assays.

2.4. *Quantitative real-time reverse transcription PCR (qRT-PCR)*

Total RNA was isolated by RNeasy Micro kit according to the manufacturer's instructions (Qiagen, Dusseldorf, Germany). Then RNA was treated with DNase I (Promega, Fitchburg, WI, USA), and used Superscript III (Invitrogen) to generate complementary DNA (cDNA). The amount of cDNA for each gene was analyzed by quantitative real-time reverse transcription PCR (qRT-PCR; ABI7900, Applied Biosystems, Carlsbad, CA, USA) with gene specific primers and SYBR GREEN 2x master mix (KAPA Biosystems, Wilmington, MA, USA). All results were normalized against the mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GADPH). The sequences of qRT-PCR primers used were as follows: CCL5 forward, 5'-CGC TGT CAT CCT CAT TGC TA-3'; reverse, 5'-GAG CAC TTG CCA CTG GTG TA-3'; ALP forward, 5'-TGG AGC TTC AGA AGC TCA

ACA CCA-3'; reverse, 5'-ATC TCG TTG TCT GAG TAC CAG TCC-3'; BSP forward, 5'-GAG AAT ACC ACA CTT TCT GCT AC-3'; reverse, 5'-AAG TAG CTG TAC TCA TCT TCA TAG G-3'; OPN forward, 5'-GCC AAA ATA GAG CTG CCT TG-3'; reverse, 5'-GTC ATG GCT TTC GTT GGA CT-3'; DLX5 forward, 5'-GAG AAG GTT TCA GAA GAC TCA GTA-3'; reverse, 5'-CTA GAA CAG CAA AAC ACA GTA GTC-3'; GADPH forward, 5'-CAT CAC CAT CTT CCA GGA GC-3'; reverse, 5'-ATG CCA GTG AGC TTC CCG TTC-3'.

2.5. *Western blot*

Western blot was performed as previously described (*11*). Specific antibodies against Runx2 (Santa Cruz biotechnology, CA, USA), CCL5 (Santa Cruz biotechnology), β-actin (Sigma) were used. Densitometry was performed by Image J software, and the expressed of various proteins were all normalized against the loading level of actin.

2.6. *Statistical analyses*

All statistical data are presented as the mean ± standard deviation (S.D.) of at least three biological replicates. Statistically significant differences were assessed by Student's unpaired two-tailed *t*-test. *p*-values < 0.05 were considered to represent significant differences.

3. Results and Discussion

3.1. *CCL5 plays an essential role in the osteogenic differentiation of human MSCs*

CCL5 is a chemokine that is well known for playing roles in inflammation, cancer progression, and wound healing. The interplay between inflammation and bone remodeling has drawn a lot of attention recently. It has been established that some chemokines and chemokine receptors are involved in cell fate determination or affect the cell numbers of osteoblasts or osteoclasts. Since whether CCL5 expression will be increased upon osteogenic differentiation has never been explored, we analyzed the CCL5 expression levels at different time points upon osteogenic differentiation. In these assays, human primary bone marrow MSCs were incubated with osteogenic differentiation medium consisting of dexamethasone, L-ascorbic acid, and β-glycerophosphate, and the samples were collected at different time points. As shown in Figure 1A, the expression of CCL5 was increased gradually during the process of osteogenic differentiation and the induction folds at day 7 and 10 were 1.7- and 5-fold, respectively (Figure 1A). In addition, the protein expression of CCL5 expression was also increased (Figure 1B). Because of the expression levels of CCL5 increased significantly upon osteogenic induction, we performed

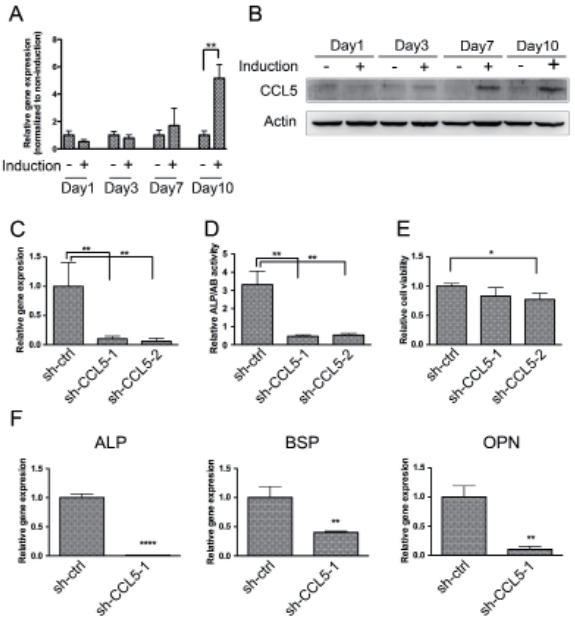

Figure 1. CCL5 plays an essential role in osteogenesis. (A) Expression of CCL5 increased upon osteogenic differentiation of human mesenchymal stem cells in a time dependent manner. hMSCs were cultured in osteogenic induction medium for 1, 3, 7 and 10 days. CCL5 mRNA levels were increased around day 7 and 10 after osteo-induction. CCL5 mRNA gene expression in cells were examined by quantitative-reverse transcription PCR (qRT-PCR) and the relative expression levels were normalized against the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GADPH) and hMSCs without induction. **(B)** The protein expression of CCL5 was increased during osteogenesis. Cell lysates were collected from different time-point of hMSCs during osteogenesis and the protein expression amounts were determined by Western blot analysis. Actin was loading control. **(C)** shRNAs of CCL5 downregulated the expression of CCL5. hMSCs were infected with lentiviruses of two independent shRNAs targeting CCL5 (shCCL5-1 or shCCL5-2), or control shRNA targeting RFP (sh-ctrl), respectively. The multiplicity of infection was 10. Various hMSCs were independently induced into osteogenic differentiation for 7 days. The CCL5 mRNA expression was examined by quantitative-reverse transcription PCR (qRT-PCR) and the relative expression level was normalized against the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GADPH) and the cells infected with sh-ctrl. **(D)** The inhibition of CCL5 expression hampered the ALP activity upon osteo-induction. ALP activities were measured and normalized against the relative cell number after cells were infected with shRNAs against CCL5 in the osteogenesis (ALP/AB ratio). **(E)** The relative cell number was not changed upon CCL5 knockdown during the process of osteogenesis. The relative cell number upon CCL5 knockdown during the process of osteogenesis was measured by Alamar blue assay. **(F)** The blockage of CCL5 expression downregulated the mRNA expression levels of osteogenic markers ALP, BSP, and OPN. The RNA expression of osteogenic markers ALP, BSP, and OPN were measured by qRT-PCR. Biological triplicates were performed in all experiments. Results are shown as the mean ± S.D. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.*p*-values were the results of applying the Student's *t*-test.

loss-of-function experiments using a shRNA lentivirus technology to evaluate the functional roles of CCL5. The knockdown efficiencies of CCL5 shRNAs in hMSCs were examined. Human MSCs were infected with two independent shRNAs, sh-CCL5-1 and sh-CCL5-2, the expression levels of CCL5 were reduced by ~90% compared with control cells (Figure 1C). The

activity of osteogenic marker, alkaline phosphatase (ALP) during the osteogenic differentiation of MSCs was dramatically decreased within CCL5-knockdown hMSCs (Figure 1D) without concomitant changes in cell number (Figure 1E). In addition, knockdown of CCL5 also repressed the expression levels of three osteoblastic markers, such as ALP, bone sialoprotein (BSP), and osteopontin (OPN) (Figure 1F). These observations indicate that CCL5 is required for the osteoblast differentiation from human MSCs into osteoblastic-committed cells.

3.2. *CCL5 promotes the osteogenesis of human MSCs*

Although we have discovered the essential role of CCL5 in the osteogenesis, the effect of exogenous CCL5 in the osteogenesis of any MSC has not been reported before. We stimulated hMSCs with human recombinant CCL5 during differentiation to investigate whether exogenous CCL5 can further promote the osteogenesis of MSCs. ALP activity was increased in CCL5 supplemented osteogenic MSCs on 7 days compared to solvent control in a dose-dependent manner (Figure 2A). This outcome does not due to any alteration of cell number of hMSCs since the cell number was not affected by CCL5 treatment (Figure 2B). Consistently, the expression levels of three osteogenic markers, ALP, distal-less homeobox 5 (DLX5), and BSP were up-regulated by CCL5 during the osteogenesis of hMSCs (Figure 2C). Additionally, the protein expression of Runt-related transcription factor 2 (RUNX2) was significantly increased in CCL5-treated osteogenic differentiation of hMSCs (Figure 2D). Our experiments provide the first evidence that CCL5 is sufficient to trigger the osteogenesis of MSCs. Taken together, these data indicate besides the known functions of CCL5 in cancer, cardiovascular disease and inflammation, we found CCL5 is essential and sufficient to promote the progression of osteogenesis of human primary MSCs.

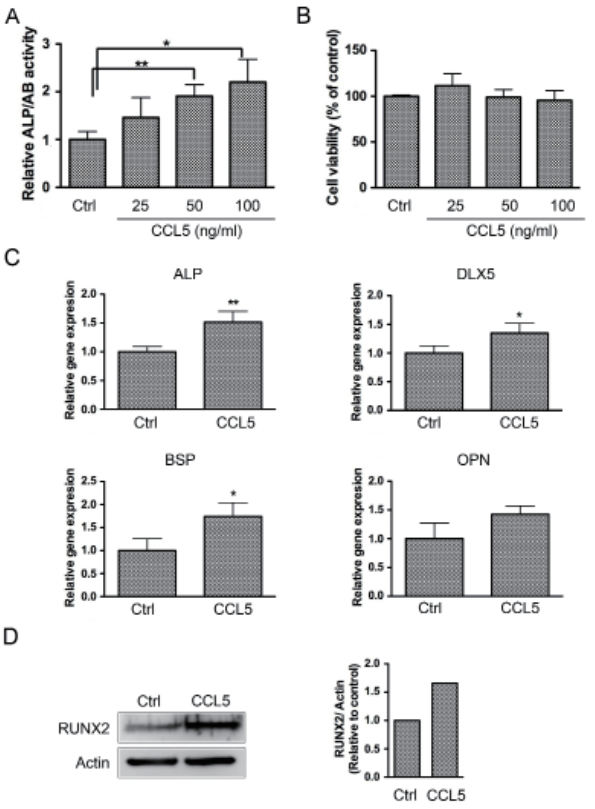

Figure 2. CCL5 promotes osteogenesis in human MSCs. hMSCs were treated with solvent control (ctrl) or different doses of CCL5 upon osteo-induction for 7 days. **(A)** ALP activity increased upon CCL5 treatment. ALP activities were measured and normalized against the relative cell number (ALP/AB ratio) after cells were treated with CCL5 in the osteogenic induction medium. **(B)** The relative cell number was not affected by CCL5 treatment during the process of osteogenesis. Alamar blue activity was measured. **(C)** The expression profile of osteogenic-related markers were measured in hMSCs treated by CCL5 during osteogenesis. Cells were incubated in the osteogenic differentiation medium with CCL5 (100 ng/mL) and RNA was collected. Relative expression levels of mRNA were determined by qRT-PCR, which were normalized against the expression levels of GAPDH and solvent control (ctrl). **(D)** The protein level of Runx2 was increased in MSCs treated by CCL5 during osteogenesis. (Left panel) hMSCs were treated with CCL5 and the RUNX2 expression amounts were determined by Western blot analysis. Actin was loading control. (Right panel) The induction fold of RUNX2 protein normalized with actin. Biological triplicates were performed in all experiments. Results are shown as the mean ± S.D. *, $p < 0.05$; **, $p < 0.01$; NS, non significant. *p*-values were the results of applying the Student's *t*-test.

3.3. *CCL5 receptor CCR1 is critical for the osteogenic differentiation of hMSCs*

CCR1, one of the CCL5 receptors, was the only receptor abundantly expressed in human MSCs (*9*). Since CCL5 plays an important role in osteogenesis (Figures 1 and 2), then we further examine whether CCR1 was required for osteogenesis of hMSCs. CCR1 was knocked down in hMSCs by the infection with shRNA lentiviruses. We found that ALP activity decreased significantly in CCR1 knockdown hMSCs (Figure 3A). Of note, the cell number was also dramatically reduced when hMSCs were infected with shRNAs against CCR1 (Figure 3B). These data suggest that CCR1 is both critical for the osteogenesis of hMSCs and maintain the cell numbers from hMSCs differentiate into osteo-committed cells. In contrast, CCL5 knockdown did not reduce the cell numbers (Figure 2). To explain why CCR1 but not CCL5 knockdown affects the cell numbers during the process of osteogenesis, one reason might be because CCR1 signaling can be activated by several different chemokines in addition to CCL5, such as chemokine (C-C motif) ligand 4 (CCL4), chemokine (C-C motif) ligand 6 (CCL6), chemokine (C-C motif) ligand 14 (CCL14), chemokine (C-C motif) ligand 15 (CCL15), chemokine (C-C motif) ligand 16 (CCL16) and chemokine (C-C motif) ligand 23 (CCL23) (12-15). There might have other chemokines in addition to CCL5 secreted by hMSCs upon osteogenic induction or present in the

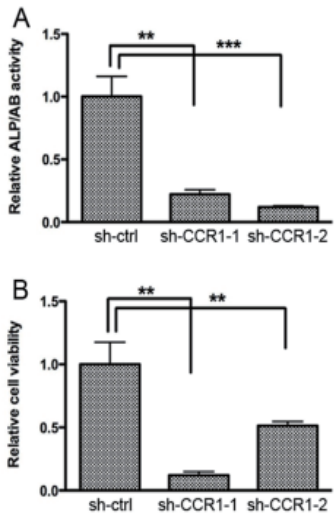

Figure 3. The knockdown of CCR1 expression inhibited osteogenic differentiation of hMSCs and decreased the relative cell numbers. hMSCs were infected with lentiviruses of two independent shRNAs targeting CCR1 (shCCR1-1 or shCCR1-2) or control shRNA targeting RFP (sh-ctrl) respectively. hMSCs were induced into osteogenic differentiation for 7 days. The multiplicity of infection was 10. **(A)** The downregulation of CCR1 blocked osteogenesis as revealed by the downregulation of ALP activity. ALP activities were measured and normalized against the relative cell number (ALP/AB ratio). **(B)** The knockdown of CCR1 downregulated the relative cell numbers during the process of osteogenesis. Alamar blue activity assays were performed to measure relative cell numbers. Biological triplicates were performed in all experiments. Results are shown as the mean ± S.D. **, $p < 0.01$; ***, $p < 0.001$. *p*-values were the results of applying the Student's *t*-test.

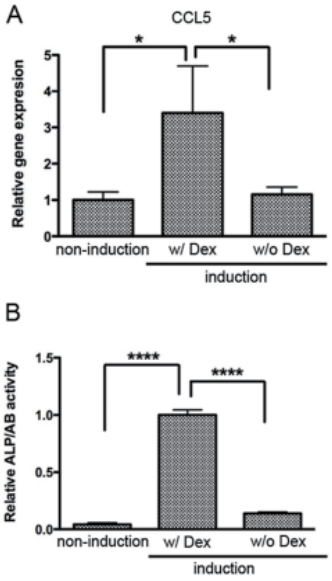

Figure 4. The expression of CCL5 was regulated by dexamethasone. (A) The induction of CCL5 expression upon osteogenic differentiation was dependent upon the presence of dexamethasone (Dex). hMSCs were cultured in expanded or osteogenic induction medium which contained with dexamethasone (w/Dex) or without dexamethasone (w/o Dex) for 7 days. The mRNA level of CCL5 was assessed by quantitative-reverse transcription PCR (qRT-PCR). The relative expression levels of CCL5 were normalized against the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GADPH) and hMSC without induction. **(B)** ALP activity decreased in the osteo-induction medium without dexamethasone. ALP activities were measured and normalized against the relative cell number (ALP/AB ratio) after cells were incubated in osteo-induction medium with or without dexamethasone. Biological triplicates were performed in all experiments. Results are shown as the mean ± SD.Results are shown as the mean ± S.D. *, $p < 0.05$; ****, $p < 0.0001$. *p*-values were the results of applying the Student's *t*-test.

fetal calf serum that play a role in maintaining cell numbers during the process of osteogenesis. Thus only knockdown of CCR1 but not CCL5 is sufficient to affect the cell numbers during the process of osteogenesis.

3.4. *The expression of CCL5 is regulated by dexamethasone*

The mechanism of regulation of CCL5 expression in osteogenesis is also unknown. Dexamethasone is a glucocorticoid hormone that is well known for its role to induce osteoblastic differentiation in human MSCs when applied it at low dose (*16*). To identify an upstream regulator of CCL5, next, we checked whether dexamethasone would affect the expression of CCL5 in hMSCs during osteogenesis. We examined the expression levels of CCL5 in the osteogenic differentiation medium with or without dexamethasone. We found that the induction of CCL5 was blocked in the absence of dexamethasone (Figure 4A). Without dexamethasone, human MSCs could not differentiate into osteo-lineage cells, which is revealed by the decrease of ALP activity (Figure 4B). This observation suggests that dexamethasone is one of the important upstream regulators of CCL5 during osteogenesis. However, our finding is the opposite of previous reports that showed dexamethasone inhibits CCL5 expression in mouse calvarial osteoblasts (*7*). One possible explanation is that there is a difference between mouse and human cells in osteoblast induction. The other hypothesis is these experimental observations were simply made at different differentiation stages, that CCL5 was only induced by dexamethasone early when hMSC differentiate into pre-osteoblast, but this induction does not occur during the late stages of pre-osteoblast differentiate into osteoblast. Although the role of dexamethasone is well-known for promoting osteogenesis in human MSCs (*16*); the effect of dexamethasone in osteogenesis is controversial *in vivo*. Dexamethasone might promote the osteoporosis (*17*), but also shown beneficial effects in bone healing (*18*). Moreover, dexamethasone was reported induce Runx2 expression by FHL2/β-catenin-mediated transcriptional activation and upregulation of TAZ and MKP1(*19*). In this paper, we found that Runx2 was increased by CCL5 treatment. Therefore, dexamethasone may activate the CCL5/CCR1 pathway signal and mediate Runx2 signal (*19*).

In summary, these results demonstrate that CCL5 and its receptor CCR1 are essential for osteogenesis of hMSCs, and that CCL5 expression is regulated

by dexamethasone. Moreover, CCL5 can promote osteogenesis as revealed by increasing ALP activity and elevating ALP, DLX5, and BSP gene expression. CCL5 has been shown to associate with STAT3 in an autocrine-loop in breast cancer cells (*20*), and STAT3 was also shown to be involved in osteogenesis (*21*). Therefore, our future studies will examine whether there is an association between STAT3 and CCL5 that is crucial for osteogenesis of hMSCs.

Acknowledgements

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Brief Report

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Serum levels of soluble carbonic anhydrase IX are decreased in patients with diffuse cutaneous systemic sclerosis compared to those with limited cutaneous systemic sclerosis

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Summary **Hypoxia may play an important role in the pathogenesis of systemic sclerosis (SSc). Carbonic anhydrase IX (CA IX) is one of the hypoxia markers and its extracellular domain can be released into the serum. However, the clinical significance of serum CA IX levels in SSc is still unknown. The aim of this study is to evaluate the possibility that serum CA IX levels can be a specific disease marker of SSc. Serum samples were obtained from SSc patients and healthy controls. Patients diagnosed as scleroderma spectrum disorder (SSD), who did not fulfill the ACR criteria of SSc but were thought that they might develop SSc in the future, were also included in this study. Serum CA IX levels were measured with specific enzyme-linked immunosorbent assays. SSD patients had significantly lower CA IX levels than diffuse cutaneous SSc (dcSSc), limited cuntaneous SSc (lcSSc) and healthy control groups. Also, we found a significant decrease in the values in dcSSc patients compared to those of lcSSc patients. Serum levels of CA IX may be useful for the differentiation of lcSSc from SSD. Decreased serum CA IX levels in spite of the presence of hypoxia in SSc may indicate an impaired response to hypoxia, which leads to the persistent hypoxic condition. Our results suggest that the abnormal response to hypoxia may already exist in SSD patients, and may be involved in its pathogenesis.**

Keywords: Carbonic anhydrase IX, collagen disease, hypoxia, systemic sclerosis

1. Introduction

Systemic sclerosis (SSc) or scleroderma is an acquired disorder which typically results in fibrosis of the skin and internal organs. Although the pathogenesis of this disease is still unclear, it includes inflammation, autoimmune attack, and vascular damage, leading to activation of fibroblasts and abnormal accumulation of extracellular matrix, mainly collagen (*1,2*).

Microangiopathy is one of the primary pathologic components of SSc (*3*). Raynaud's phenomenon or aberrant nailfold bleeding is known as an early vascular event of this disease. Telangiectasia, pitting scars, skin ulcers, impaired wound healing or pulmonary hypertension are frequently observed in the disease process, and they can severely affect the quality of life in these patients.

The microangiopathy causes a reduction of blood flow, which results in tissue hypoxia. The tissue ischemia leads usually to the expression of angiogenic growth factors, which act against the ischemic conditions. Hypoxia induced factor (HIF)-1α, one of the hypoxic markers, is a transcription factor which regulates cellular adaptation to low oxygen tension (*4*). Under normoxic conditions, the expression of HIF-1α is maintained at a low level by ubiquitination and degradation (*5*). In hypoxic conditions, HIF-1α is up-regulated and translocated to the nucleus where it induces transcription of target genes essential for survival and adaptation to hypoxic environments, such as vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT-1), and erythropoietin (*6*).

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Cutaneous hypoxia in patients with SSc was reported (*7*). Distler O *et al*. describe that despite severely reduced oxygen levels, protein levels of HIF-1α in the skin of SSc patients were even below the levels seen in healthy control skin (*8*). Therefore, the impaired response to tissue hypoxia in SSc patients may lead to persistence of hypoxic conditions. Hypoxia contributes directly to progression of fibrosis by activation of fibroblasts in SSc. On the other hand, excess extracellular matrix deposition increases diffusion distances from blood vessels to cells, and induces further hypoxic conditions (*9*). This vicious circle is thought to be associated with the pathogenesis of SSc.

In this study, we focused on carbonic anhydrase IX (CA IX), another hypoxia marker. Carbonic anhydrases (CAs) are a family of zinc-containing enzymes, that catalyze a reversible conversion of carbon dioxide to bicarbonate and a proton in the reaction: $CO_2+H_2O \leftrightarrow HCO_3^-+H^+$ (*10*). These enzymes participate in a variety of biological processes, including respiration, ion transport, pH balance, and bone resorption (*11*). Human CAs exist in at least 15 isoforms (*12*). Among them, CA IX is a membrane-associated protein and is known as a biomarker of hypoxia or certain malignant tumors. The expression of CA IX can only be detected in a few normal tissues, whereas it is abnormally induced in hypoxic conditions or malignant tumors (*13*). The relationship of CA IX with hypoxia has been explained by the notion that the CA IX promoter contains a hypoxia response element (HRE) to which HIF-1 can bind (*14*). Because the extracellular domain of CA IX can be released into cell culture medium or into the body fluids (*15*), unlike HIF-1α, CA IX can be detected in serum. As described above, hypoxia plays an important role in the pathogenesis of SSc. Although the clinical significance of serum CA IX levels in SSc is still unknown, they can be correlated with disease activity. Thus, in this study, we try to evaluate the possibility that serum levels of CA IX can be a useful marker of SSc.

2. Materials and Methods

2.1. *Clinical assessment and patient material*

Serum samples were obtained from 43 patients with SSc (7 men and 36 women; age range, 7-85 years; mean, 57.4 years). All patients fulfilled the criteria proposed by the American College of Rheumatology, and were grouped according to the classification system proposed by LeRoy *et al*. (*16*): 20 patients had diffuse cutaneous SSc (dcSSc) and 23 patients had limited cutaneous SSc (lcSSc), as described previously (*17*). Clinical and laboratory data reported in this study were obtained at the time of serum sampling. Patients were evaluated for the presence of gastrointestinal, pulmonary, cardiac, renal or muscle involvement as described previously (*17*). Control serum samples were also collected from healthy age- and sex-matched volunteers. Five patients diagnosed as scleroderma spectrum disorder (SSD), who did not fulfill the ACR criteria of SSc but were thought that they might develop SSc in the future based on the criteria proposed by Ihn *et al*., were also included in this study (*18-20*). Institutional review board approval and written informed consent were obtained before patients and healthy volunteers were entered into this study according to the Declaration of Helsinki. All serum samples were stored at –80°C prior to use.

2.2. *Measurement of serum CA IX concentrations*

Levels of serum CA IX were measured with a specific ELISA kit (R&D Systems, Minneapolis, MN, USA). Briefly, anti-CA IX monoclonal antibodies were precoated onto microtiter wells. Aliquots of serum were added to each well, followed by peroxidase-conjugated antibodies to CA IX. Color was developed with hydrogen peroxide and tetramethylbenzidine peroxidase and the absorbance at 450 nm was measured. Wavelength correction was performed by absorbance at 570 nm. The concentration of CA IX in each sample was determined by interpolation from a standard curve.

2.3. *Statistical analysis*

Statistical analysis was carried out with a Welch two sample *t*-test for the comparison of means, and Fisher's exact probability test for the analysis of frequency. *p* values less than 0.05 were considered significant.

3. Results and Discussion

The serum CA IX levels in patients with SSc and in healthy control subjects are shown in Figure 1. Serum samples were obtained from 43 patients with SSc. Twelve healthy control subjects and 5 SSD patients, who did not fulfill the criteria of SSc but were thought that they might develop SSc in the future, were also included in this study. The SSD patients had 4-8 points using a point system proposed by Ihn *et al*. (*20*).

Although mean serum CA IX levels were higher in SSc patients (146 ± 198 pg/mL) than in healthy control subjects (118 ± 115 pg/mL), there was no statistically significant difference between the two groups. However, when SSc patients were classified into lcSSc and dcSSc as described in 'Patients and Methods', we found a significant decrease in the values of dcSSc patients than in those of lcSSc patients (82 ± 68 *vs.* 201 ± 254 pg/mL, $p < 0.05$). The mean serum levels were higher in lcSSc patients and lower in dcSSc patients than those in healthy controls, but there were no significant difference.

On the other hand, CA IX levels in all 5 SSD patients were decreased as compared to other groups;

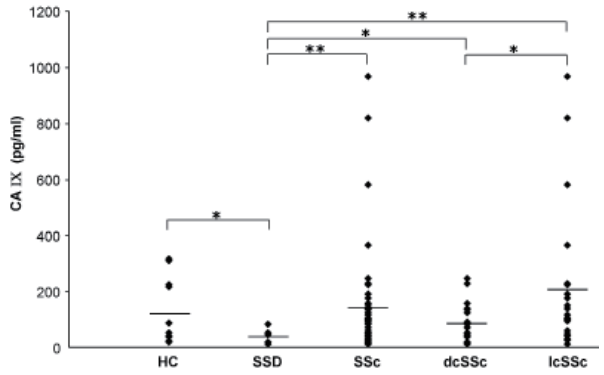

Figure 1. Serum concentrations of soluble CA IX in patients with systemic sclerosis (SSc), scleroderma spectrum disorders (SSD), and in healthy control subjects (HC). Serum CA IX levels were measured with ELISA kits as described in 'Materials and Methods'. Serum CA IX concentrations are shown on the ordinate. Bars show means. p values less than 0.05 are interpreted as significant. dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc. $^{*}p < 0.05$, $^{**}p < 0.01$.

SSD patients had significantly lower CA IX levels than healthy controls (42 ± 28 *vs*. 118 ± 114 pg/mL, $p < 0.05$) and SSc patients (42 ± 28 *vs*. 146 ± 198 pg/mL, $p < 0.01$). In addition, the difference between SSD patients and lcSSc patients (42 ± 28 *vs*. 201 ± 254 pg/mL, $p < 0.01$) was more significant than that between SSD patients and dcSSc patients (42 ± 28 *vs*. 82 ± 68 pg/mL, $p < 0.05$). Taken together, the serum CA IX levels were decreased in patients with SSD and dcSSc in that order, with statistical significance.

Table 1 shows the association of serum CA IX levels with the clinical features in SSc patients. Considering that HIF-1α expression in the skin of SSc patients was previously reported to be below the levels seen in healthy control skin (*8*), and that the serum CA IX levels in SSD and dcSSc patients tended to be decreased in our study, we regarded reduction of CA IX levels as the meaningful change in SSc patients. As shown in Table 1, in SSc patients with reduced CA IX levels, the percentage of dcSSc was significantly increased compared to those with normal CA IX levels (dcSSc:lcSSc = 14:8 *vs*. 6:15, $p = 0.022$). There was no statistically significant difference between these groups in terms of sex, mean age at onset, duration of disease, and other clinical or laboratory features including therapy, smoking history, respiratory dysfunction or anemia.

In this study, although we expected that hypoxia marker CA IX was up-regulated in SSc sera due to its hypoxic conditions, there was no significant difference in serum CA IX between healthy controls and SSc patients. However, we found that SSD patients had significantly lower CA IX levels than control subjects, dcSSc or lcSSc patients. In addition, serum CA IX levels were significantly lower in dcSSc patients compared to lcSSc patients.

The concept of SSD was originally proposed by

Table 1. Correlation of serum CA IX levels with clinical features in patients with systemic sclerosis (SSc)

Items	Patients with normal CA IX levels (n =21)	Patients with reduced CA IX levels (n =22)
Sex (female : male)	18:3	18:4
Type (diffuse : limited)	6:15*	14:8*
Clinical Features		
Raynaud's phenomenon	76.2	90.9
Pitting scar	47.6	36.4
Ulcer	33.3	27.3
Nailfold bleeding	33.3	50.0
Cobblestone on hands	4.8	13.6
Hyperkeratotic plaque	0	9.1
Telangiectagia	23.8	18.2
Contracture of phalanges	66.7	72.7
Calcinosis	4.8	0
Inflammatory erythema	95.2	45.5
Diffuse pigmentation	19.0	18.2
Short subungal flenurum	52.4	45.5
Sicca symptoms	47.6	18.2
Organ involvement		
Oesophagus	19.0	18.2
Ileus	0	0
Pulmonary fibrosis	23.8	50.0
Heart	52.4	13.6
Liver	19.0	13.6
Kidney	0	0
Joint	23.8	34.8
Thrombosis	0	0
Thyroiditis	28.6	27.3
Others		
Complication of malignancy	0	0

Unless noted otherwise, values are percentages. Fisher's exact probability test was performed to compare the frequency of clinical findings of patients. P values less than 0.05 were considered significant. $^{*}p < 0.05$.

Maricq *et al*. to unify typical SSc, early forms of SSc and closely related disorders including mixed connective tissue disease (MCTD) (*18*). Thereafter, Ihn *et al*. defined SSD as patients who did not fulfill the criteria of SSc but were thought that they might develop SSc in the future, and established a new diagnostic method using a points system to distinguish patients with SSD from those with early SSc. A total score was obtained as the sum of the following five factors: (1) extent of skin sclerosis (maximum, 10 points); (2) pulmonary changes (maximum, 4 points); (3) antinuclear antibodies (maximum, 5 points); (4) pattern of Raynaud's phenomenon (maximum, 3 points); and (5) nailfold bleeding (maximum, 2 points). The authors suggest the conditions with 9 or more points are consistent with SSc and those with 5 to 8 points are consistent with SSD (*20*). Because progressive fibrosis of SSc is often irreversible, at least clinically, there is an urgent need to develop new strategies to diagnose patients as early as possible and follow them carefully. For that purpose, the concept of SSD should be further understood and characterized. Our study is the first to perform ELISA experiments using SSD sera.

To note, there were significant difference between

SSD patients and lcSSc patients. The diagnosis of SSc presents little problem when the clinical features have fully developed. However, it may be difficult to distinguish lcSSc from SSD, because skin sclerosis is sometimes not apparent in lcSSc, especially in a very early stage. Serum levels of CA IX may be useful for the differentiation of lcSSc from SSD. Moreover, we frequently encounter SSD patients with an increased risk of future development of SSc. Serial time-course measurement of serum CA IX concentration in SSD patients may lead to early detection of developing SSc.

As described above, CA IX is thought to be a down-stream target of HIF-1α (*14*). Thus, reduced CA IX levels seen in dcSSc patients in our study is consistent with down-regulation of HIF-1α in SSc skin in spite of the presence of hypoxia (*8*), which may result in the persistent hypoxic condition of the disease. Our results suggest that such dysregulation of HIF-1α as well as CA IX and subsequent persistent hypoxia may already occur in SSD patients. Considering that microangiopathy is one of the primary symptoms of SSc and tissue fibrosis is observed in the disease process, the impaired response to microangiopathy-induced hypoxic conditions may be involved in the pathogenesis of SSD. Furthermore, a sustained abnormal response may lead to the development of severe skin sclerosis in dcSSc patients, whereas the reduction of CA IX may be transient in lcSSc. Otherwise, the discrepancy between dcSSc and lcSSc in CA IX levels may indicate heterogeneity between the two clinical subtypes.

There are some limitations to our results. First, we could not collect large number of SSD patients because of the rarity of this condition. However, our approach may be effective to clarify the properties of SSD. Larger studies are needed in the future. In addition, recent studies show transforming growth factor (TGF)-β, one of the key cytokines in the pathogenesis of SSc, can also regulate CA IX gene expression (*21*). Thus, there may be another pathway other than HIF-1α in the regulation of CA IX. The regulatory mechanisms of CA IX in this disease should be clarified.

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Original Article

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Evaluation of human D-amino acid oxidase inhibition by antipsychotic drugs *in vitro*

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Summary **It is of importance to determine whether antipsychotic drugs currently prescribed for schizophrenia exert D-amino acid oxidase (DAO)-inhibitory effects. We first investigated whether human (h)DAO can metabolize D-kynurenine (D-KYN) to produce the fluorescent compound kynurenic acid (KYNA) by using high-performance liquid chromatography with mass spectrometry, and fluorescence spectrometry. After confirmation of KYNA production from D-KYN by hDAO, 8 first- and second-generation antipsychotic drugs, and 6 drugs often prescribed concomitantly, were assayed for hDAO-inhibitory effects by using *in vitro* fluorometric methods with D-KYN as the substrate. DAO inhibitors 3-methylpyrazole-5-carboxylic acid and 4*H*-thieno[3,2-b]pyrrole-5-carboxylic acid inhibited KYNA production in a dose-dependent manner. Similarly, the second-generation antipsychotics blonanserin and risperidone were found to possess relatively strong hDAO-inhibitory effects *in vitro* (5.29 ± 0.47 μM and 4.70 ± 0.17 μM, respectively). With regard to blonanserin and risperidone, DAO-inhibitory effects should be taken into consideration in the context of their *in vivo* pharmacotherapeutic efficacy.**

Keywords: Schizophrenia, D-kynurenine, D-serine, second-generation antipsychotic drug, risperidone, blonanserin

1. Introduction

Schizophrenia, a serious neuropsychiatric disease, affects 1% of the general population and consists of positive symptoms, negative symptoms, and cognitive impairments (*1*). Current therapeutics include first-generation (typical) antipsychotics (FGA) and second-generation (atypical) antipsychotics (SGA) (*2*), and selecting the most suitable antipsychotic is crucial for improving the quality of daily life for patients with schizophrenia.

Inhibition of D-amino acid oxidase (DAO) (EC 1.4.3.3.) (*3-5*) is considered to be an effective pharmacotherapy for schizophrenia. It has been reported that treatment of schizophrenia patients with D-serine, a co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor, improved symptoms when administered in combination with antipsychotics (*6,7*). Therefore, much attention has recently been focused on the inhibition of DAO, which decomposes endogenous D-serine *in vivo*, and selective DAO inhibitors have been developed and investigated by pharmaceutical company research groups (*8-10*).

Despite this research focus on DAO inhibition, the primary pharmacological action of most institutionally prescribed antipsychotics is the blockade of dopamine D2 and serotonin receptors (*2*); however, these drugs might also inhibit DAO activity *in vivo*. Therefore, an evaluation of the DAO-inhibitory effects of antipsychotics presently prescribed in medical institutions is important because their efficacy might

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be partly due to increased endogenous D-serine levels. Recently, we reported a fluorescence assay method for the evaluation of pig kidney (pk) DAO inhibition by a drug using D-kynurenine (D-KYN) as a substrate. This assay has the advantage of simple and facile operation because it measures the fluorescence intensity of a fluorescent compound, kynurenic acid (KYNA), that is produced from D-KYN by DAO through a single enzymatic reaction (*11,12*). Thus, in the present study, this *in vitro* assay was applied to human DAO (hDAO) and was used to evaluate the DAO-inhibitory effects of currently prescribed antipsychotic drugs, as well as of concomitant drugs prescribed to schizophrenia patients.

2. Materials and Methods

2.1. *Materials*

Quetiapine fumarate (Seroquel® tablets) and aripiprazole (Abilify® tablets) were purchased from Astellas Pharma Inc. (Tokyo, Japan) and Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Other drugs were purchased from Sigma Co. Ltd. (St. Louis, MO, USA), Tokyo Chemical Industries Co. Ltd. (Tokyo, Japan), and Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). Recombinant hDAO was prepared according to previously described methods (*3, 13,14*), and the hDAO-inhibition assay was performed with D-KYN as a substrate, using methods similar to our previous paper (*12*).

2.2. *DAO assay with D-KYN as a substrate*

A detailed procedure for the measurement of the DAO-inhibitory effects of test drugs was described in our previous papers (*12*). Briefly, we mixed 20 μL of 0.1 mg/mL DAO in 0.4 M Tris buffer solution (pH 8.3), 50 μL of 200 μM FAD solution, 20 μL of 2.0 mg/mL BSA in H_2O, 370 μL of 0.4 M Tris buffer solution (pH 8.3), and 20 μL of tested drugs or compounds dissolved in DMSO, and incubated this mixture at 37°C for 20 min. Next, 7.0 mM D-KYN (20 μL) was added, and the mixture was incubated at 37°C for 60 min. In the case of determination of Km, varying concentrations (0.1, 0.2, 0.4, 0.8, 1.75 mM) of D-KYN (20 μL) were added in the presence or absence of the tested drugs, and incubated at 37°C for 60 min. The reaction mixture was dissolved in H_2O with 1,500 μL of 0.4 M Tris buffer solution (pH 8.3) and 50 μL of 300 mM $ZnSO_4$. The final solution was subjected to vortex mixing, and the fluorescence of the solution was measured by a HITACHI F-7000 fluorescence spectrometer (Hitachi Co. Ltd., Tokyo, Japan). The fluorescence intensity of each solution was measured at an excitation wavelength of 250 nm and emission wavelength of 394 nm. According to the following equation (1), the ΔF value was determined and used to calculate kinetic parameters (K_m) using the Lineweaver-Burk plot:

$$\Delta F = F - F_0 \quad (1)$$

where F and F_0 are fluorescence intensities of the sample and blank sample (a sample treated without D-KYN), respectively. For the inhibition curve, the final concentrations of the test compounds were plotted on the X-axis, and the ΔF value in the absence of the inhibitor was set as 100% on the Y-axis. The degree of inhibition for each test compound was expressed as a percentage according to the following equation (2):

$$\text{Remaining activity of hDAO (\%)} = \Delta F' / \Delta F \times 100 \quad (2)$$

where $\Delta F'$ is the ΔF value at each concentration of the inhibitor. IC_{50} values of the tested drugs were calculated using the following equation (3):

$$IC_{50} = 10\ [\log (A/B) \times (50-C)/(D-C) + \log B] \quad (3)$$

where A and B are the higher and lower concentrations near 50% inhibition, respectively, and C and D are the inhibition percentages at B and A, respectively.

2.3. *LC-MS analysis*

After enzymatic reaction with DAO for 60 min, the reactant was subjected to ultra-filtration with a Centrifree® (EMD Millipore Corporation, Billerica, MA, USA), without the addition of Tris-buffer and $ZnSO_4$. The ultra-filtration was carried out at 2,000 g for 15 min at room temperature. For a blank sample, 0.4 M Tris buffer (pH 8.3) was added to the mixture instead of DAO solution. The filtrate was vortex-mixed with the mobile phase, and 20 μL of the filtrate was injected into the LC-MS apparatus, comprising an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, U.S.A.) and a time-of-flight (TOF)-MS (JMS-T100LP AccuTOF LC-Plus) equipped with an electrospray ionization (ESI) source (JEOL Co. Ltd., Tokyo, Japan). The separation column was a TSKgel ODS-100V (150 mm × 2.0 mm; *i.d.*: 5 μm; Tosoh Corporation, Tokyo, Japan) equipped with a guard column, and the mobile phase was 10 mM HCO_2NH_4 in H_2O (pH 3.5)/MeOH (80:20). The mobile phase was constantly pumped at 0.20 mL/min, and the column temperature was maintained at 45°C. The conditions for ESI-MS detection were as follows: positive ion mode, needle voltage set at 2,000 V, and ring lens and orifice 1 and 2 voltages set at 10, 70, and 10 V, respectively. Nitrogen was used as the nebulizing and desolvation gas, and the pressure was maintained at 0.608 MPa. The desolvation chamber and orifice 1 temperatures were set to 250°C and 80°C, respectively. Data were obtained using Mass Center software, MS-56010MP (JEOL).

2.4. *Fluorescence spectra*

Excitation and emission spectra of the reactant solution were measured by a HITACHI F-7000 fluorescence spectrometer at 15, 30, 45, and 60 min after onset of the enzymatic reaction of D-KYN with hDAO. Each sample was prepared and treated in a similar manner as described in section 2.2.

3. Results and Discussion

3.1. *KYNA production by hDAO*

In a previous paper, we reported a fluorescence assay for the evaluation of pkDAO-inhibitory effects of a drug by utilizing an enzymatic reaction of D-KYN as a DAO substrate to produce KYNA (Figure 1) (*11,12*). The enzymatic product, KYNA, emits fluorescence at 398 nm (ex. 250 nm) in the presence of Zn (II), and the attenuation of fluorescence intensity originating from KYNA can be used for the evaluation of the DAO-inhibitory effect of a drug. However, results obtained using hDAO have not been reported. Some differences in physical properties and substrate specificity between pkDAO and hDAO have been reported by Molla *et al*. (*5*). In the present study, our previous assay was altered to use hDAO, and we evaluated the hDAO-inhibitory effects of currently prescribed antipsychotic drugs. Firstly, the enzymatic production of KYNA from D-KYN by hDAO was investigated using high-performance liquid chromatography with UV and mass spectrometric detection (LC-UV-MS), and measurement of the fluorescence spectrum.

Figure 2 shows enzymatic reactant chromatograms and MS spectra obtained by LC-UV-MS. In the chromatogram of enzymatic reactant (Figure 2c), a peak corresponding to the retention time of KYNA was found, and the mass spectrum was consistent with KYNA (Figures 2a and 2c).

Next, a time-dependent generation of fluorescence due to KYNA in the enzymatic reactant was investigated using fluorescence spectroscopy. Figure 3 shows the time-course of excitation and fluorescence spectra of the reaction mixture of D-KYN with hDAO. A fluorescence intensity at 398 nm, which is the wavelength for KYNA, increased with reaction time. The excitation and emission spectra of the product generated by the enzymatic reaction of hDAO with D-KYN were consistent with

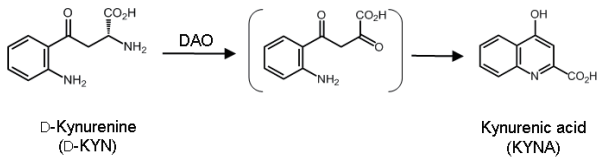

Figure 1. Transformation of D-kynurenine (D-KYN) to kynurenic acid (KYNA) by DAO.

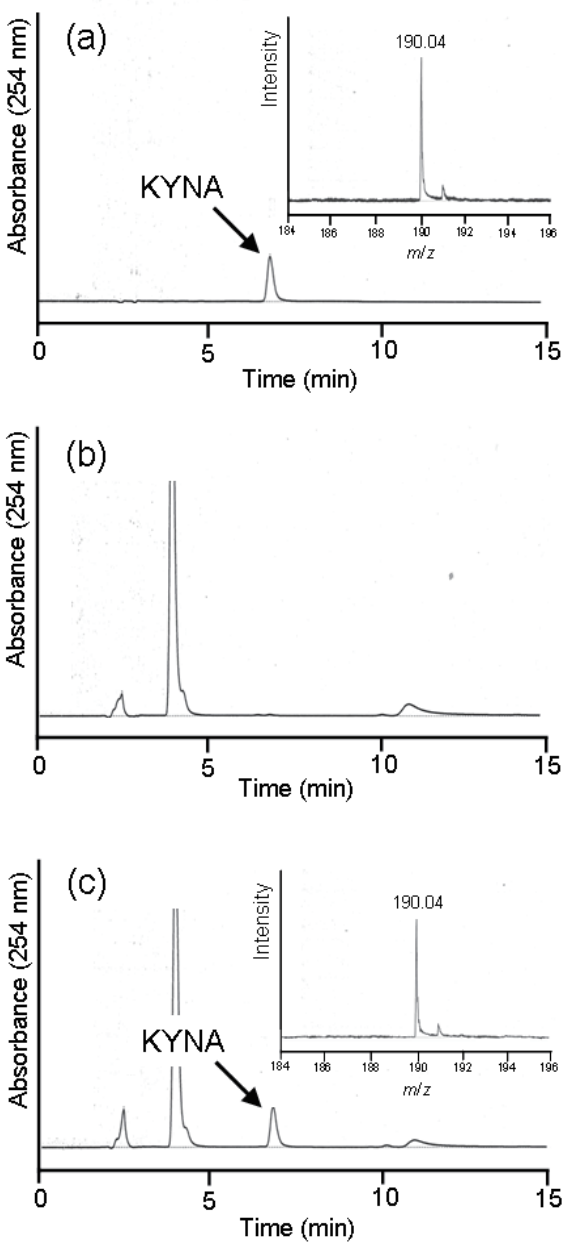

Figure 2. Chromatograms of standard KYNA (20 μM) (a), blank sample without hDAO (b), and enzymatic reactant of D-KYN with hDAO at 60 min (c). The insets in **(a)** and **(c)** are mass spectra of standard KYNA and the peak indicated by the arrow, respectively, obtained by LC-UV-MS.

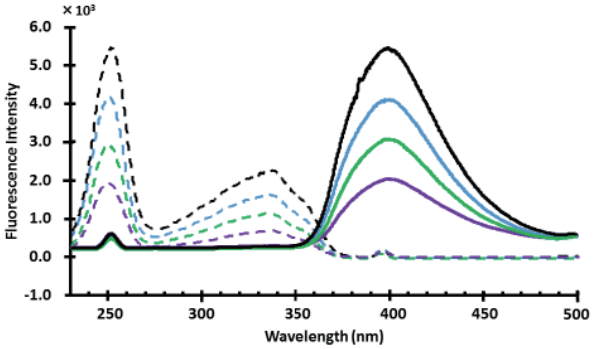

Figure 3. Excitation (dotted line) and emission spectra (solid line) of the reaction solution of D-KYN with hDAO at 15 (purple), 30 (green), 45 (blue), and 60 min (black), respectively.

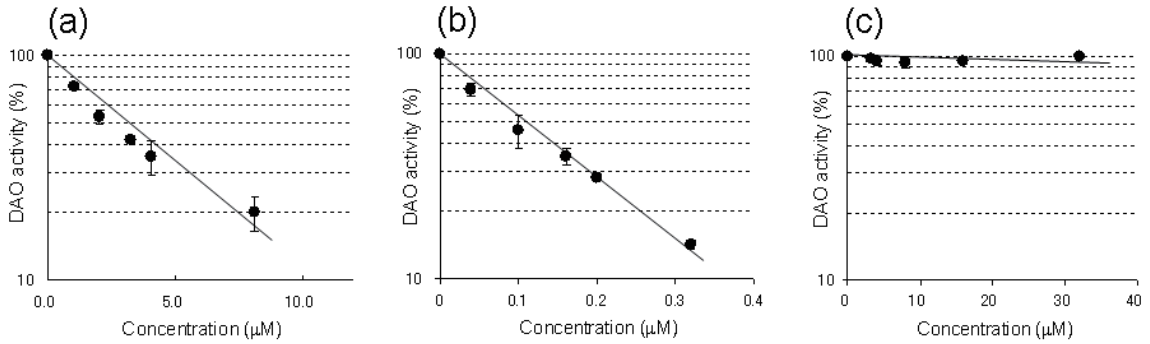

Figure 4. Dose-inhibition curves for 3-methylpyrazole-5-carboxyl acid (MPC) (circle), 4*H*-thieno[3,2-b]pyrrole-5-carboxylic acid (compound 8) (triangle), and 3-methylpyrazole-4-carboxyl acid (3,4-MPC) (square). Human D-amino acid oxidase (DAO) inhibition by MPC and compound 8 was clearly observed.

those of KYNA (*15*), and the increase in fluorescence intensity was dependent on reaction time. This result indicated that, like pkDAO, hDAO can metabolize D-KYN to KYNA (*11,12*).

Based on these results, it was concluded that KYNA was produced from D-KYN by hDAO. The K_m and V_{max} values of D-KYN determined using the Lineweaver-Burk plots were 1.78 mM and 5.88 μmol/(min·mg), respectively. Considering that the reported Km values of D-Ser and D-Ala were 41 and 3.1 mM, respectively (*5*), it may be considered that D-KYN has a moderate affinity to hDAO.

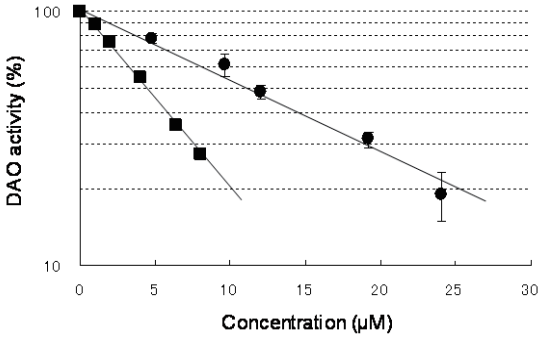

Figure 5. Dose- inhibition curves for chlorpromazine-(circle) and risperidone (square)-mediated human D-amino acid oxidase (DAO) inhibition.

3.2. *Inhibition of DAO activity*

Under the enzymatic reaction conditions described earlier, two DAO inhibitors reported on previously, 3-methylpyrazole-5-carboxylic acid (MPC, AS057278) (*16*) and 4*H*-thieno[3,2-b]pyrrole-5-carboxylic acid (Compound 8) (*17*) were tested for enzyme inhibition. As shown in Figures 4a and 4b, both inhibitors inhibited KYNA production in a dose-dependent manner, indicating that the proposed *in vitro* assay can be used for the evaluation of hDAO activity in a similar manner to that of pkDAO. The IC_{50} values for MPC and compound 8 were 2.41 ± 0.23 μM and 90.3 ± 18.1 nM, respectively. Compound 8 exhibited a more intense DAO-inhibitory effect than MPC, which was consistent with previously reported results (*9*).

In contrast, little inhibition of human DAO was observed due to addition of 3-methylpyrazole-4-carboxylic acid (3, 4-MPC), a structural isomer of MPC (Figure 4c). This result was similar to previously published results using pkDAO (*12*).

Next, the *in vitro* fluorescence assay using D-KYN as a substrate was used to determine the hDAO-inhibitory effects of FGAs (chlorpromazine, haloperidol, and sulpiride) and SGAs (risperidone, olanzapine, aripiprazole, quetiapine, and blonanserin). In addition, other drugs given to schizophrenia patients were also tested, including anti-parkinson drugs (trihexyphenidyl, biperiden), anti-depressant drugs (sertraline, escitalopram, duloxetine), and the mood stabilizer valproate (unpublished data). Figure 5 shows dose-inhibition curves representative of chlorpromazine and risperidone that were obtained using the proposed fluorescence assay. Attenuation of hDAO activity in a concentration-dependent manner was observed with both drugs, indicating that considerable inhibition of hDAO activity was caused, and that more intense inhibition of hDAO was produced by risperidone than by chlorpromazine. The 50% inhibitory concentration (IC_{50}) values of test drugs obtained using the proposed assay are summarized in Table 1. Several drugs exhibited IC_{50} values for hDAO-inhibitory effects below 50 μM. Among them, the second-generation antipsychotics blonanserin and risperidone were found to possess relatively strong hDAO-inhibitory effects *in vitro* (5.29 ± 0.47 and 4.70 ± 0.17 μM, respectively). These IC_{50} values were comparable to that (2.41 ± 0.23 μM, $n = 3$) of MPC, a compound with established DAO-inhibitory activity. The precise mechanism responsible for hDAO-inhibition is not clear, but chlorpromazine and risperidone have been reported to inhibit hDAO (*13,18,19*). The common features of the tested drugs were a heterocyclic moiety and a tertiary amino group. In the case of chlorpromazine, replacement of flavin adenine dinucleotide, a co-factor of DAO, occurred that might have resulted in attenuated DAO activity (*19-*

Table 1. IC_{50} values (μM) of FGAs, SGAs, and concomitant drugs for the inhibition of hDAO activity by the proposed assay (mean ± S.D., *n* = 3-4)

Drug	IC_{50}
FGA	
Chlorpromazine	11.6 ± 0.58
Haloperidol	13.4 ± 1.87
Sulpiride	14.3 ± 0.81
SGA	
Aripiprazole	12.4 ± 1.38
Blonanserin	5.29 ± 0.47
Olanzapine	> 50.0
Quetiapine	43.9 ± 9.27
Risperidone	4.70 ± 0.17
Anti-parkinson	
Trihexyphenidyl	> 50.0
Biperiden	> 50.0
Anti-depressant	
Sertraline	> 50.0
Escitalopram	> 50.0
Duloxetine	> 50.0
Mood stabilizer	
Valproate	> 50.0

21). Thus, it may be reasonable to conclude that the hDAO-inhibitory effects of drugs in this study were at least partially due to the presence of a heterocyclic moiety and a tertiary amino group, similar to those in chlorpromazine. However, it is conceivable that the structure of a compound can be strictly discriminated by the active site of DAO, because various IC_{50} values for each drug were revealed (Table 1). To clarify the precise mechanism by which each drug inhibited DAO activity, co-crystallographic analyses of DAO with each compound or drug will be necessary.

The present data suggest that prescribed anti-psychotic drugs might inhibit hDAO activity *in vivo*. From surveillance data collected by the Japanese Psychiatric Clinical Pharmacy Research Group (*22*), poly-pharmacy of anti-psychotic drugs, particularly SGAs, occurs in medical institutions in Japan. Therefore, drug combination therapies consisting of SGAs might enhance inhibition of DAO in the central nervous system. In the future, the DAO-inhibitory effect of SGAs should be taken into consideration with regard to their prescription.

In summary, by using the assay proposed herein, we revealed that SGAs blonanserin and risperidone possess hDAO-inhibitory effects *in vitro*. In addition to primary pharmacological actions such as blockade of dopamine D_2 and serotonin receptors, the DAO-inhibitory effects of these drugs may contribute to their pharmacotherapeutic efficacy.

Acknowledgements

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Original Article

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Comparison of mechanisms underlying changes in glucose utilization in fasted rats anesthetized with propofol or sevoflurane: Hyperinsulinemia is exaggerated by propofol with concomitant insulin resistance induced by an acute lipid load

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Summary **The effects of anesthesia with sevoflurane and with propofol on glucose utilization in rats were investigated. Sevoflurane significantly impairs glucose utilization whereas propofol does not. Both insulin secretion and sensitivity affect glucose utilization. Propofol is hydrophobic, and anesthesia with this agent is always accompanied by an acute lipid load, which can exaggerate insulin resistance. The role of the acute lipid load in the effects of anesthesia with sevoflurane and propofol on glucose utilization in fasted rats was investigated. Rats were allocated to groups anesthetized with sevoflurane and infused with physiological saline (group S) or 10% w/v lipid (group SL), or those anesthetized with propofol (group P). Intravenous glucose tolerance tests and insulin tolerance tests were then performed to measure glucose utilization, and blood glucose, plasma insulin, and plasma TNF-α levels were measured. In the intravenous glucose tolerance test, groups SL and P showed significantly higher plasma insulin levels than group S, and group P showed significantly higher plasma insulin levels than group SL. In the insulin tolerance test, groups SL and P showed insulin resistance compared to group S, but no significant difference was observed between groups SL and P. In summary, propofol anesthesia enhances insulin secretion and concomitantly exaggerates insulin resistance, compared with sevoflurane anesthesia. Propofol appears to be the main cause of hyperinsulinemia, and the acute lipid load exaggerates insulin resistance.**

Keywords: General anesthesia, insulin secretion, insulin sensitivity, lipid metabolism, tumor necrosis factor-α

1. Introduction

We previously investigated the effects of anesthesia with different agents on glucose metabolism in rats and found that sevoflurane impairs glucose utilization, while propofol does not (*1*). Volatile anesthetics, such as sevoflurane, activate adenosine triphosphate-sensitive potassium channels in β-islet cells, resulting in attenuated insulin secretion (*2-5*). Although the precise mechanisms have not been elucidated, insulin secretion is significantly enhanced in rats under propofol anesthesia (*6,7*). Plasma insulin levels and insulin sensitivity regulate glucose utilization. Our recent findings suggested that insulin sensitivity is significantly impaired by propofol anesthesia compared with sevoflurane anesthesia (*7*). Due to the hydrophobic properties of propofol, a lipid formulation is generally used for anesthesia, and this imposes an acute lipid load. Recent studies (*8-11*) have shown that an acute lipid load exaggerates insulin resistance. Therefore, the role of an acute lipid load in the effects of anesthesia with sevoflurane and propofol on insulin secretion and sensitivity were investigated in fasted rats.

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2. Materials and Methods

2.1. *Subjects*

The Animal Care Committee of The University of Tokyo approved the experimental protocols (Approval number: H13-047) in this study (Figures 1 and 2). Nine- to 10-week-old, male Wistar rats were housed in a regulated environment at an ambient temperature of 25°C under a 12-hour light-dark cycle (7 AM and 7 PM). Water and a standard diet comprised of 24% protein, 5% fat, 6% ash, 3% fiber, 8% water, and 54% nitrogen-free extract were provided *ad libitum*, and all rats were fasted for 12 h before starting the study. Hypothermia was prevented during the experiments using a heat lamp and a heating pad.

2.2. *Surgical preparation*

Anesthesia was induced with 5% sevoflurane (Maruishi Pharmaceutical Co. Ltd., Osaka, Japan) in 1.5 L/min oxygen administered *via* a tightly fitting face mask in 42 rats during surgical preparation. All rats underwent tracheotomy and tracheal intubation. Sevoflurane (2.5% in 0.5 L/min oxygen) was administered *via* the tracheal tube, and the lungs were mechanically ventilated at a tidal volume of 2.5 mL and a respiratory rate of 55 breaths/min. A 19-gauge catheter was inserted into the right carotid artery, and another catheter was inserted into the right jugular vein. Catheter patency was maintained with 100 IU of intravenous heparin. Hemodynamic variables were recorded, and 1.5 mL of arterial blood was sampled immediately after surgical preparation (T1).

2.3. *Intravenous glucose tolerance test (IVGTT)*

The administered doses of sevoflurane and propofol for maintenance of anesthesia were selected based on our previous protocols (*1,6,7*). Twenty-one rats were assigned to groups S-IVGTT, SL-IVGTT, and P-IVGTT (n = 7 in each group; Figure 1). Sevoflurane anesthesia was continued in groups S-IVGTT and SL-IVGTT. In group S-IVGTT, physiological saline was administered intravenously with a 4 mL/kg bolus, followed by continuous infusion at a rate of 4 mL/kg/h. In group SL-IVGTT, 10% w/v Intralipid (Fresenius Kabi Japan K.K., Tokyo, Japan) was administered intravenously with a 4 mL/kg bolus, followed by continuous infusion at a rate of 4 mL/kg/h. In group P-IVGTT, sevoflurane administration was discontinued, and instead, 10 mg/mL of propofol solution (1% Diprivan; AstraZeneca K.K., Osaka, Japan) was administered intravenously with a 4 mL/kg bolus, followed by continuous infusion at a rate of 4 mL/kg/h. After stabilization for 30 min, 1 g/kg of glucose was administered intravenously to all rats for the IVGTT. Hemodynamic variables were recorded, and 1.5 mL of arterial blood was sampled, immediately before (T2) and at 15 (T3) and 30 (T4) min after glucose administration. The lipid loads were equal in groups SL-IVGTT and P-IVGTT.

2.4. *Insulin tolerance test (ITT)*

The administered doses of sevoflurane and propofol were selected as described above. Another 21 rats were assigned to groups S-ITT, SL-ITT, and P-ITT (n = 7 in each group; Figure 2), surgically prepared as described above, and given 10% glucose at a rate of 10 mL/kg/h intravenously. Sevoflurane anesthesia was continued in groups S-ITT and SL-ITT, which then received intravenously a 4 mL/kg bolus of physiological saline or 10% w/v intralipid, followed by a continuous infusion at a rate of 4 mL/kg/h of physiological saline or 10% w/v intralipid, respectively. In group P-ITT, sevoflurane administration was discontinued, and instead, 1% diprivan was administered intravenously with a 4 mL/kg bolus, followed by continuous infusion at a rate of 4 mL/kg/h. After stabilization for 30 min, 10 IU/kg of the rapid-acting human insulin analogue (Humulin-R; Eli Lilly Japan K.K., Hyogo, Japan) was administered intravenously to all groups for the ITT (*12-14*). Hemodynamic variables were recorded, and 1.5 mL of arterial blood was sampled immediately before (T2) and at 15 (T3) and 30 (T4) min after insulin administration. The lipid loads were equal in groups SL-ITT and P-ITT.

2.5. *Measurements*

The arterial catheter was connected to a low-volume pressure transducer to monitor mean arterial blood pressure (MAP) and heart rate (HR). Immediately after each blood sampling, blood glucose and β-hydroxybutyrate (β-OHB) levels were measured using Medisafe (Terumo, Tokyo, Japan) and Precision Xceed (Abbott Japan Co. Ltd., Tokyo, Japan), respectively. Blood samples were spun in a prerefrigerated centrifuge (4°C) at 1000× g for 15 min, and plasma specimens were stored at -60°C. Plasma insulin and TNF-α levels were measured using AKRIN-010T and AKRTN-010 enzyme-linked immunosorbent assays, respectively (Shibayagi Co. Ltd., Gunma, Japan).

The quantitative insulin sensitivity check index (QUICKI) was calculated using the following equation: QUICKI = 1/(log [plasma insulin level (μIU/mL)] + log [blood glucose level (mg/dL)]) (*15*). The area under the time-response curve above the glucose level at T2 in each rat was calculated to evaluate changes in blood glucose levels during the IVGTT (AUC [T2-T4]). In addition, Δglucose [T2-T4] was calculated to evaluate changes in blood glucose levels during the ITT using the following equation: Δglucose [T2-T4] (mg/dL) = [blood glucose level at T4 (mg/dL)] - [blood glucose level at T2 (mg/dL)].

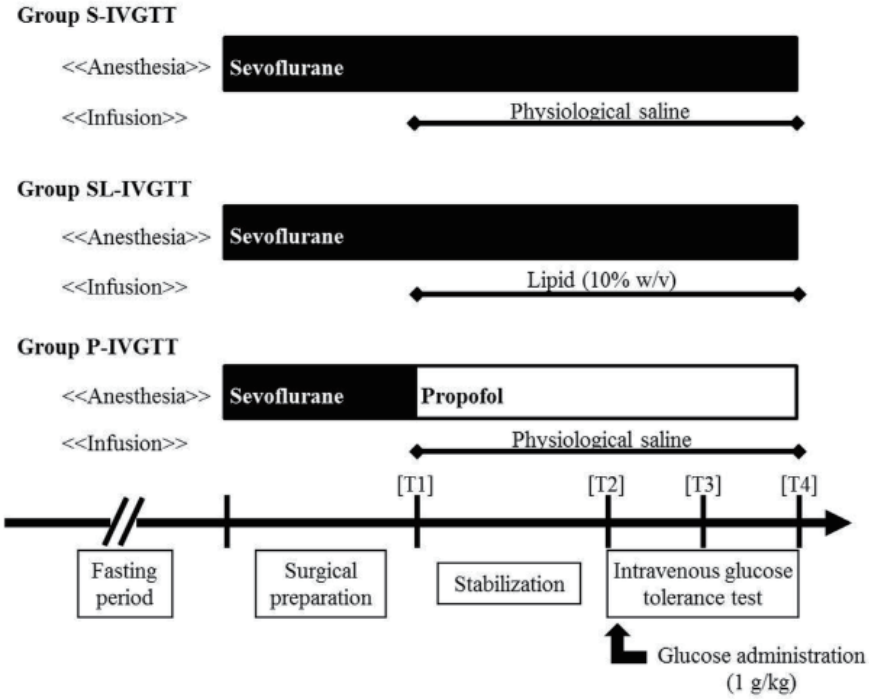

Figure 1. Experimental protocols for intravenous glucose tolerance tests (IVGTT). A set of 21 rats underwent surgical preparation under sevoflurane anesthesia. Rats were assigned to 3 groups: groups S-IVGTT, SL-IVGTT, and P-IVGTT. Sevoflurane anesthesia was continued in groups S-IVGTT and SL-IVGTT. Physiological saline was administered intravenously in group S-IVGTT, while 10% w/v lipid was administered intravenously in group SL-IVGTT. Sevoflurane administration was discontinued, and instead propofol solution was administered intravenously in group P-IVGTT. After a 30-min stabilization period, the intravenous glucose tolerance test was performed. Hemodynamic variables were recorded, and arterial blood was sampled immediately after surgical preparation (T1), immediately before (T2), and at 15 min (T3) and 30 min (T4) after glucose administration.

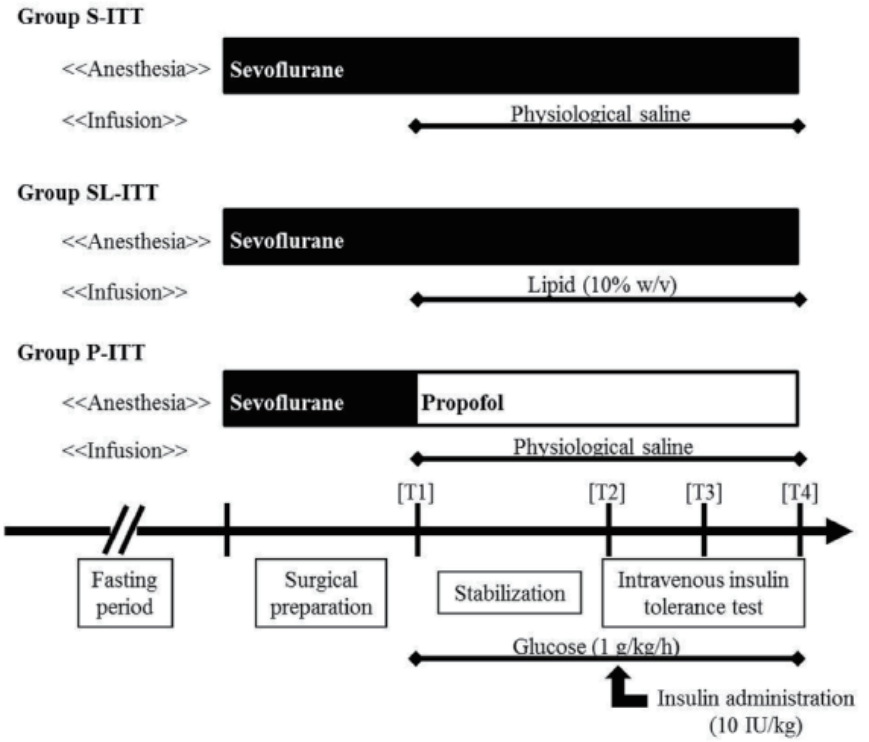

Figure 2. Experimental protocols for insulin tolerance tests (ITT). Another set of 21 rats underwent surgical preparation under sevoflurane anesthesia. Rats were assigned to 3 groups: groups S-ITT, SL-ITT and P-ITT. Immediately after surgical preparation, all rats were administered glucose by continuous infusion. Sevoflurane anesthesia was continued in groups S-ITT and SL-ITT. Physiological saline was administered intravenously in group S-ITT, while 10% w/v lipid was administered intravenously in group SL-ITT. Sevoflurane administration was discontinued, and instead propofol solution was administered intravenously in group P-ITT. After a 30-min stabilization period, the insulin tolerance test was performed. Hemodynamic variables were recorded, and arterial blood was sampled, immediately after surgical preparation (T1), immediately before (T2), and at 15 min (T3) and 30 min (T4) after insulin administration.

Table 1. Hemodynamic parameters during intravenous glucose tolerance tests in groups S-IVGTT, SL-IVGTT, and P-IVGTT

Items	T1	T2	T3	T4
Mean arterial blood pressure (mmHg)				
S-IVGTT	90 ± 19	81 ± 13	85 ± 7	67 ± 17
SL-IVTT	89 ± 6	90 ± 6	90 ± 9	74 ± 9
P-IVGTT	85 ± 11	111 ± 25*	93 ± 26	66 ± 33
Heart rate (beats/min)				
S-IVGTT	367 ± 21	399 ± 24	386 ± 28	372 ± 33
SL-IVGTT	380 ± 37	410 ± 38	390 ± 35	388 ± 32
P-IVGTT	375 ± 41	408 ± 53	372 ± 33	352 ± 29

Data are shown as means ± S.D. T1: just after surgical preparation. T2: just before glucose administration. T3: 15 min after glucose administration. T4: 30 min after glucose administration. Two-way repeated-measures ANOVA detected a significant difference among the three groups in the time course of MAP, but not in the time course of HR. *: adjusted $p < 0.05$ *versus* group S-IVGTT at the same time point, one-way ANOVA with the Bonferroni-Dunn test.

2.6. *Statistics*

Data were statistically analyzed using JMP Pro version 10.0.2. (SAS Institute, Cary, NC, USA). Parametric data are shown as means ± S.D. Serial data were compared among three groups using two-way repeated-measures analysis of variance (ANOVA) with group and time points as the factors; statistical significance was set at $p < 0.05$. Sphericity was checked using Mauchly's test; statistical significance was set at $p < 0.05$. When sphericity was not met, the Greenhouse-Geisser correction was applied; statistical significance was set at an adjusted $p < 0.05$. Parametric data were compared among three groups at each time point using one-way ANOVA; statistical significance was set at $p < 0.05$. When a significant difference was noted, the Bonferroni-Dunn test was applied for multiple comparisons; statistical significance was set at an adjusted $p < 0.05$.

Insulin and TNF-α levels in plasma and QUICKI are shown as medians [25th and 75th percentiles]. Non-parametric data at each time point were compared among three groups using the Kruskal-Wallis test; statistical significance was set at $p < 0.05$. When a significant difference was detected, the Steel-Dwass test was used for multiple comparisons; statistical significance was set at an adjusted $p < 0.05$.

3. Results

3.1. *IVGTT results*

The weights of the rats in groups S-IVGTT, SL-IVGTT, and P-IVGTT did not differ significantly: 273 ± 22, 271 ± 18, and 287 ± 26 g, respectively.

Table 1 shows the time course of the hemodynamic parameters during IVGTT in groups S-IVGTT, SL-IVGTT, and P-IVGTT. There was a significant difference in the time course of MAP among the three groups ($p = 0.0052$). MAP differed significantly among the three groups at T2 ($p = 0.0075$); group P-IVGTT showed significantly higher MAP than group S-IVGTT (adjusted $p = 0.0072$). There was no significant difference in the time course of HR among the three groups.

Table 2. Changes in blood glucose levels during intravenous glucose tolerance tests in groups S-IVGTT, SL-IVGTT, and P-IVGT

Items	Blood glucose levels (mg/dL)				AUC [T2-T4] (min•mg/dL)
	T1	T2	T3	T4	
S-IVGTT	79 ± 19	74 ± 17	246 ± 13	170 ± 15#	3301 ± 470
SL-IVGTT	87 ± 15	70 ± 9	217 ± 25	144 ± 22*	2763 ± 471
P-IVGTT	94 ± 18	53 ± 17*	196 ± 25*	113 ± 13*#	2601 ± 301*

Data are shown as means ± S.D. AUC [T2-T4]: the area under the time-response curve of blood glucose levels during the intravenous glucose tolerance test above the blood glucose level at T2 in each rat. T1: just after surgical preparation. T2: just before glucose administration. T3: 15 min after glucose administration. T4: 30 min after glucose administration. Two-way repeated-measures ANOVA with Greenhouse-Geisser correction detected a significant difference among the three groups in the time course of blood glucose levels. *: adjusted $p < 0.05$ versus group S-IVGTT at the same time point, one-way ANOVA with the Bonferroni-Dunn test. #: adjusted $p < 0.05$ versus group SL-IVGTT at the same time point, one-way ANOVA with the Bonferroni-Dunn test.

Table 2 shows the time course of blood glucose levels during the IVGTT in groups S-IVGTT, SL-IVGTT, and P-IVGTT. There was a significant difference in the time course of blood glucose levels among the three groups (adjusted $p < 0.0001$). Blood glucose levels differed significantly among the three groups at T2, T3, and T4 ($p = 0.0358$, $= 0.0020$ and < 0.0001, respectively). Group P-IVGTT showed significantly lower blood glucose levels at T2 and T3 than group S-IVGTT (adjusted $p = 0.0480$ and $p = 0.0015$, respectively). Groups SL-IVGTT and P-IVGTT showed significantly lower blood glucose levels at T4 than group S-IVGTT (adjusted $p = 0.0376$ and < 0.0001, respectively), and when compared to group SL-IVGTT, group P-IVGTT showed significantly lower blood glucose levels (adjusted $p = 0.0112$). AUC [T2-T4] differed significantly among the three groups ($p = 0.0164$). Group P-IVGTT showed significantly lower AUC [T2-T4] than group S-IVGTT (adjusted $p = 0.0195$).

Table 3 shows the time course of blood β-OHB levels during the IVGTT in groups S-IVGTT, SL-IVGTT, and P-IVGTT. There was a significant difference in the time

course of blood β-OHB levels among the three groups (adjusted $p < 0.0001$). Blood β-OHB levels differed significantly at T2, T3, and T4 among the three groups ($p = 0.0073$, < 0.0001, and < 0.0001, respectively). Group P-IVGTT showed significantly lower blood β-OHB levels at T2 than group SL-IVGTT (adjusted $p = 0.0066$). Group SL-IVGTT showed significantly higher blood β-OHB levels at T3 than groups S-IVGTT and P-IVGTT (adjusted $p < 0.0001$ and $= 0.0001$, respectively). Groups SL-IVGTT and P-IVGTT showed significantly higher blood β-OHB levels at T4 than group S-IVGTT (adjusted $p < 0.0001$ and $= 0.0001$, respectively), and when compared to group SL-IVGTT, group P-IVGTT showed significantly lower blood β-OHB levels (adjusted $p = 0.0283$, respectively).

Table 3. Changes in blood β-hydroxybutyrate levels during intravenous glucose tolerance tests in groups S-IVGTT, SL-IVGTT, and P-IVGTT

Items	T1	T2	T3	T4
Blood glucose levels (mg/dL)				
S-IVGTT	1.7 ± 0.6	2.1 ± 0.9	0.7 ± 0.5#	0.1 ± 0.1#
SL-IVGTT	1.8 ± 0.5	3.0 ± 0.5	2.3 ± 0.5*	1.5 ± 0.4*
P-IVGTT	1.5 ± 0.6	1.7 ± 0.6#	1.1 ± 0.5#	0.9 ± 0.4*#

Data are shown as means ± SD. T1: just after surgical preparation. T2: just before glucose administration. T3: 15 min after glucose administration. T4: 30 min after glucose administration. Two-way repeated-measures ANOVA with Greenhouse-Geisser correction detected a significant difference among the three groups in the time course of blood β-hydroxybutyrate levels. *: adjusted $p < 0.05$ versus group S-IVGTT at the same time point, one-way ANOVA with the Bonferroni-Dunn test. #: adjusted $p < 0.05$ versus group SL-IVGTT at the same time point, one-way ANOVA with the Bonferroni-Dunn test.

Table 4 shows the time course of plasma insulin levels during the IVGTT in groups S-IVGTT, SL-IVGTT, and P-IVGTT. Plasma insulin levels differed significantly among the three groups at T2, T3, and T4 ($p = 0.0001$, $p = 0.0001$, and $p = 0.0002$, respectively). Groups SL-IVGTT and P-IVGTT showed significantly higher plasma insulin levels at T2, T3, and T4 than group S-IVGTT (adjusted $p = 0.0061$ in all comparisons), and when compared to group SL-IVGTT, group P-IVGTT showed significantly higher plasma insulin levels at T2, T3, and T4 (adjusted $p = 0.0061$, $= 0.0061$, and $= 0.0137$, respectively).

QUICKI at T1 in groups S-IVGTT, SL-IVGTT, and P-IVGTT was 0.338 [0.309, 0.346], 0.315 [0.304, 0.329], and 0.304 [0.283, 0.319], respectively; QUICKI at T1 did not differ significantly among the three groups. QUICKI at T2 in groups S-IVGTT, SL-IVGTT, and P-IVGTT was 0.345 [0.310, 0.350], 0.253 [0.251, 0.257], and 0.241 [0.228, 0.253], respectively; QUICKI at T2 differed significantly among the three groups ($p = 0.0005$). Groups SL-IVGTT and P-IVGTT showed significantly lower QUICKI at T2 (adjusted $p = 0.0061$ and $p = 0.0061$, respectively), whereas QUICKI at T2 was similar between groups SL-IVGTT and P-IVGTT.

3.2. *ITT results*

The weight of the rats did not differ significantly among the S-ITT, SL-ITT, and P-ITT groups: 287 ± 19, 278 ± 14, and 291 ± 12 g, respectively.

Table 5 shows the time course of hemodynamic parameters during the ITT in groups S-ITT, SL-ITT, and

Table 4. Changes in plasma insulin levels during intravenous glucose tolerance tests in groups S-IVGTT, SL-IVGTT, and P-IVGTT

Items	T1	T2	T3	T4
Plasma insulin levels (μIU/mL)				
S-IVGTT	13 [12, 16]	12 [12, 17]§	122 [102, 251]§	44 [30, 75]§
SL-IVGTT	18 [14, 19]	122 [118, 131]†	538 [503, 650]†	300 [168, 420]†
P-IVGTT	20 [18, 35]	259 [249, 378]†§	3683 [2372, 4853]†§	2531 [702, 3447]†§

Data are shown as medians [25th, 75th percentiles]. T1: just after surgical preparation. T2: just before glucose administration. T3: 15 min after glucose administration. T4: 30 min after glucose administration. †: adjusted $p < 0.05$ versus group S-IVGTT at the same time point, Kruskal-Wallis test with the Steel-Dwass test. §: adjusted $p < 0.05$ *versus* group SL-IVGTT at the same time point, Kruskal-Wallis test with the Steel-Dwass test.

Table 5. Hemodynamic parameters during insulin tolerance tests in groups S-ITT, SL-ITT, and P-ITT

Items	T1	T2	T3	T4
Mean arterial blood pressure (mmHg)				
S-ITT	90 ± 13	95 ± 19	81 ± 17	69 ± 9
SL-ITT	92 ± 25	99 ± 9	78 ± 19	71 ± 14
P-ITT	82 ± 12	125 ± 14*#	96 ± 29	71 ± 33
Heart rate (beats/min)				
S-ITT	371 ± 23	408 ± 34	401 ± 26	382 ± 25
SL-ITT	369 ± 21	379 ± 34	371 ± 42	375 ± 31
P-ITT	346 ± 46	393 ± 41	377 ± 40	350 ± 46

Data are shown as means ± SD. T1: just after surgical preparation. T2: just before insulin administration. T3: 15 min after insulin administration. T4: 30 min after insulin administration. Two-way repeated-measures ANOVA detected a significant difference among the three groups in the time course of MAP, but not in the time course of HR. *: adjusted $p < 0.05$ versus group S-ITT at the same time point, one-way ANOVA with the Bonferroni-Dunn test. #: adjusted $p < 0.05$ versus group SL-ITT at the same time point, one-way ANOVA with the Bonferroni-Dunn test.

P-ITT. There was a significant difference in the time course of MAP among the three groups ($p = 0.0272$). MAP differed significantly among the three groups at T2 ($p = 0.0017$); group P-ITT showed significantly higher MAP than groups S-ITT and SL-ITT (adjusted $p = 0.0072$ and $= 0.0093$, respectively). There was no significant difference in the time course of HR among the three groups.

Table 6 shows the time course of blood glucose levels during the ITT in groups S-ITT, SL-ITT, and P-ITT. There was a significant difference in the time course of blood glucose levels among the three groups (adjusted $p < 0.0001$). Blood glucose levels differed significantly among the three groups at T2 and T4 ($p = 0.0020$ and $p = 0.0032$, respectively). Group P-ITT showed significantly lower blood glucose levels at T2 and significantly higher blood glucose levels at T4 than group S-ITT (adjusted p = 0.0015 and p = 0.0027, respectively). Δglucose [T2-T4] differed significantly among the three groups ($p < 0.0001$). Groups SL-ITT and P-ITT showed significantly less decreases in blood glucose levels after insulin administration than group S-ITT (adjusted $p = 0.0064$ and $p = 0.0001$, respectively).

Table 7 shows the time course of plasma TNF-α levels during the ITT in groups S-ITT, SL-ITT, and P-ITT. TNF-α was undetectable at T1 in all rats in the three groups. TNF-α was detected at T2 in all rats in groups SL-ITT and P-ITT, but not detected in any rats in group S-ITT. TNF-α was detected at T3 in 5 of 7 rats in group SL-ITT and in all rats in group P-ITT, but not detected in any rats in group S-ITT. TNF-α was detected at T4 in 1 of 7 rats in group S-ITT, in 4 of 7 rats in group SL-ITT, and in 6 of 7 rats in group P-ITT. Plasma TNF-α levels differed significantly among the three groups at T2, T3, and T4 ($p = 0.0009$, $= 0.0022$ and $= 0.0107$, respectively). Groups SL-ITT and P-ITT showed significantly higher plasma TNF-α levels than group S-ITT at T2 (adjusted $p = 0.0030$ and $= 0.0030$, respectively) and at T3 (adjusted $p = 0.0305$ and $p = 0.0030$, respectively). Group P-ITT showed significantly higher plasma TNF-α levels than group S-ITT at T4 (adjusted $p = 0.0063$).

Table 6. Changes in blood glucose levels during insulin tolerance tests in groups S-ITT, SL-ITT, and P-ITT

Items	Blood glucose levels (mg/dL)				Δglucose [T2-T4](mg/dL)
	T1	T2	T3	T4	
S-ITT	80 ± 12	167 ± 21	110 ± 12	95 ± 10	-72 ± 29#
SL-ITT	79 ± 17	145 ± 8	122 ± 14	130 ± 17	-15 ± 17*
P-ITT	91 ± 18	131 ± 17*	124 ± 23	151 ± 41*	21 ± 31*

Data are shown as means ± SD. Δglucose [T2-T4]: the changes in blood glucose levels during the insulin tolerance test. T1: just after surgical preparation. T2: just before insulin administration. T3: 15 min after insulin administration. T4: 30 min after insulin administration. Two-way repeated-measures ANOVA with Greenhouse-Geisser correction detected a significant difference among the three groups in the time course of blood glucose levels. *: adjusted $p < 0.05$ versus group S-ITT at the same time point, one-way ANOVA with the Bonferroni-Dunn test. #: adjusted $p < 0.05$ versus group SL-ITT at the same time point, one-way ANOVA with the Bonferroni-Dunn test.

4. Discussion

Based on the glucose levels and the changes in blood glucose levels (*i.e.*, AUC[T2-T4]) in groups S-IVGTT, SL-IVGTT and P-IVGTT, we consider that groups SL-IVGTT and P-IVGTT utilized larger amounts of glucose during the IVGTT than group S-IVGTT. However, groups SL-IVGTT and P-IVGTT showed significantly higher β-OHB levels during the IVGTT than group S-IVGTT. In addition, blood β-OHB levels during the IVGTT were significantly higher in group SL-IVGTT than in group P-IVGTT. Blood β-OHB levels correlate with the amount of gluconeogenesis *via* lipolysis (*i.e.*, fat catabolism). A recent clinical study (*16*) reported that glucose administration during surgery under sevoflurane anesthesia significantly suppresses lipolysis. Consistent with this finding, blood β-OHB levels decreased after glucose administration in group S-IVGTT in the present study. Therefore, exogenous lipid was probably utilized as an energy substrate in groups SL-IVGTT and P-IVGTT. It appears that an acute lipid load increases gluconeogenesis *via* lipolysis under sevoflurane anesthesia. Furthermore, the amount of exogenous lipid utilized as an energy substrate is larger under sevoflurane anesthesia than under propofol anesthesia. Taken together, these findings indicate that lipid metabolism is involved in the mechanisms underlying the different effects of sevoflurane anesthesia and propofol anesthesia on glucose utilization.

Insulin secretion is considered to predominantly regulate glucose utilization. Volatile anesthetics, such as sevoflurane, attenuate insulin secretion by activating

Table 7. Changes in plasma tumor necrosis factor-α levels during insulin tolerance tests in groups S-ITT, SL-ITT, and P-ITT

Items	T1	T2	T3	T4
Plasma tumor necrosis factor-α levels (pg/mL)				
S-ITT	0 [0, 0]	0 [0, 0]§	0 [0, 0]§	0 [0, 0]
SL-ITT	0 [0, 0]	1019 [747, 1386]†	604 [0, 1332]†	0 [0, 1207]
P-ITT	0 [0, 0]	1099 [819, 1954]†	1166 [667, 1707]†	646 [290, 730]†

Data are shown as medians [25th, 75th percentiles]. T1: just after surgical preparation. T2: just before insulin administration. T3: 15 min after insulin administration. T4: 30 min after insulin administration. †: adjusted $p < 0.05$ versus group S-ITT at the same time point, Kruskal-Wallis test with the Steel-Dwass test. §: adjusted $p < 0.05$ versus group SL-ITT at the same time point, Kruskal-Wallis test with the Steel-Dwass test.

adenosine triphosphate-sensitive potassium channels in β-islet cells (*2-5*). We previously found that propofol anesthesia enhances insulin secretion in rats (*6,7*). Groups SL-IVGTT and P-IVGTT showed significantly higher plasma insulin levels during IVGTT than group S-IVGTT. Interestingly, plasma insulin levels during IVGTT were significantly higher in group P-IVGTT than in group SL-IVGTT. These results suggest that an acute lipid load enhances insulin secretion under sevoflurane anesthesia by stimulating gluconeogenesis *via* lipolysis, and that insulin secretion can be additionally increased by propofol itself.

Insulin sensitivity is also considered to be a factor that regulates glucose utilization. Groups SL-IVGTT and P-IVGTT showed significantly lower QUICKI at T2 than group S-IVGTT, indicating insulin resistance in groups SL-IVGTT and P-IVGTT. An acute lipid load impairs insulin sensitivity (*8-11*), suggesting that lipid metabolism is involved in the regulation of insulin sensitivity. Cytokines and hormones derived from adipose tissues, such as TNF-α, regulate insulin sensitivity (*17,18*). The increase in plasma TNF-α levels associates with insulin resistance (*17,18*). Therefore, plasma TNF-α levels were measured during the ITT to further determine the mechanism underlying the insulin resistance in groups SL-IVGTT and P-IVGTT.

The decreases in blood glucose levels after insulin administration were significantly less in groups SL-ITT and P-ITT than in group S-ITT, reflecting insulin resistance in groups SL-ITT and P-ITT. There was no significant difference in the decreases in blood glucose levels after insulin administration between groups SL-ITT and P-ITT. Thus, it appears that an acute lipid load exaggerates insulin resistance under propofol anesthesia, while propofol does not.

Groups SL-ITT and P-ITT showed significantly higher plasma TNF-α levels during the ITT than group S-ITT. Therefore, an acute lipid load might increase TNF-α secretion from adipose tissues, leading to the insulin resistance observed in the present study. Further investigations are required to elucidate whether TNF-α is the main cause of the observed insulin resistance under propofol anesthesia, which is probably exaggerated by the acute lipid load.

Blood glucose levels should be appropriately controlled in patients undergoing surgery, because hyperglycemia is considered an independent risk factor for postoperative morbidity and mortality (*19-21*). Therefore, the significantly lower blood glucose levels under propofol anesthesia may reflect the possible advantageous effects of propofol anesthesia. On the other hand, the observed insulin resistance can be interpreted as a disadvantageous effect of propofol anesthesia on intraoperative glycemic control. The present findings are difficult to extrapolate to clinical practice, because the dose of propofol administered to rats is much larger than that administered to patients in clinical settings; the larger dose of propofol administration is accompanied by a larger acute lipid load. However, the results of the present study suggest that the effects of propofol anesthesia on insulin secretion, as well as insulin sensitivity, in clinical settings are worthy of further investigation.

This study has two major limitations. One is that all rats were anesthetized with sevoflurane for surgical preparation, and, thus, some residual effects might have altered glucose metabolism in groups P-IVGTT and P-ITT. The other is that the time course of hemodynamics differed significantly among groups S-IVGTT, SL-IVGTT, and P-IVGTT and among groups S-ITT, SL-ITT, and P-ITT. We previously reported that rats under propofol anesthesia at the same dose applied in the present study showed no changes in blood glucose levels during sigmoid colostomy (*1*), suggesting that propofol anesthesia is enough to suppress endocrine/metabolic responses to surgical stress. We did not measure plasma catecholamine levels. It is, therefore, difficult to estimate sympathetic nervous system activity during the experiments in rats used in the present study. Sympathetic nervous system activity is considered to modify glucose metabolism, and, thus, the possible impact of a significant difference in hemodynamics on glucose metabolism cannot be neglected.

In summary, propofol anesthesia enhances insulin secretion and concomitantly exaggerates insulin resistance compared with sevoflurane anesthesia. Propofol itself seemed to be the main cause of hyperinsulinemia rather than the acute lipid load, and insulin resistance was mainly attributed to the acute lipid load, which might be associated with the systemic release of TNF-α.

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Relationship between T-SPOT.TB responses and numbers of circulating CD4+ T-cells in HIV infected patients with active tuberculosis

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Summary **This study sought to evaluate the performance of the T-SPOT.TB assay for the diagnosis of active tuberculosis (TB) in human immunodeficiency virus (HIV) infected patients. One hundred confirmed HIV-infected patients with active TB and known T-SPOT.TB and CD4+ T-cell counts were enrolled in this clinical retrospective study. We found that patients with lower CD4+ T-cell counts (11-50 cells/μL) had the lowest T-SPOT.TB positive rates (50%), and patients with higher CD4+ T-cell counts (50-100 cells/μL) had the highest T-SPOT.TB positive rates (75%). However, there were no significant differences between the T-SPOT.TB positive rates of patients with different CD4+ T-cell counts (< 10, 11-50, 51-100 and > 100 cells/μL) (χ^2 = 3.7747, p = 0.287). The patients with positive TB culture results had significantly higher T-SPOT.TB positive rates (78.9%) than patients that were culture-negative (44.3%) (χ^2 = 12.8303, p < 0.001). Other variables, including gender, age, TB disease classification, HIV RNA level, and highly reactive antiretroviral therapy (HAART), had no significant effects on T-SPOT.TB positive rates. The number of spot-forming cells (SFCs) reactive with ESAT-6, CFP-10 and ESAT-6/CFP-10-specific T cells detected by T-SPOT.TB were positively correlated with the number of circulating CD4+ T-cells (r_s = 0.3791, p = 0.0001; r_s = 0.2929, p = 0.0031; r_s = 0.3345, p = 0.0007, respectively). This study suggests that the number of SFCs is strongly related to the degree of immunodeficiency, while the T-SPOT.TB positive rates are less dependent on the level of CD4+ T-cell depletion in HIV infection and active TB.**

Keywords: HIV, active tuberculosis, T-SPOT.TB

1. Introduction

Tuberculosis (TB) is one of the most common infectious diseases in the world. In 2012, there were an estimated 8.6 million people with active TB. Among these, there were 1.1 million (13%) co-infections with HIV (TB-HIV) and 320,000 active TB-HIV deaths according to a report from the World Health Organization (*1*). Patients with advanced immunodeficiency have a greater risk of active TB (*2*).

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Patients with active TB need early diagnosis and prompt treatment. Currently, the gold standard for active TB diagnosis remains the isolation of *Mycobacterium tuberculosis* positive cultures - a procedure that is time-consuming (*3*). Also, it is sometimes difficult to obtain suitable culture specimens other than sputum in HIV-infected patients suffering from extrapulmonary TB (*4,5*). Furthermore, *M. tuberculosis* culturing is often not available in some regions, and a Ziehl-Neelsen stain may take more than 10 days. There is a great need for new methods for diagnosing TB in HIV-infected subjects throughout the world, especially for patients with advanced immunodeficiency. The introduction of an interferon (IFN)-γ release assay (IGRAs) using the immunogenic and specific *M. tuberculosis* antigens early secreted antigenic target (ESAT-6) and culture

filtrate protein 10 kDa (CFP-10) for immunodiagnosis is, therefore, a potential advantage. There are two commercial forms of the IGRAs licensed for use in the developed world, including the T-SPOT.TB (Oxford Immunotec, Abingdon, UK), which is based on the enzyme-linked immunosorbent spot (ELISPOT) assay, and the whole blood-based QuantiFERON-TB Gold in-tube (QFT-IT; Cellestis, Melbourne, Australia), which uses an enzyme-linked immunosorbent assay (ELISA) to detect IFN-γ released into culture supernatants (*6*). The results of QFT-IT are impacted by peripheral CD4+ T-cell counts, as reported by previous studies, whereas similar problems for the T-SPOT.TB assay were not detected in several studies (*7-12*). It is thought by some that the T-SPOT.TB assay is less likely to be affected by low CD4+ T lymphocyte counts, as ELISPOT technology is the most sensitive method for detecting IFN-γ-secreting antigen-specific T cells derived from blood. Therefore, although the magnitude of the mitogen response was slightly lower in low CD4+ T-cell counts, responses were still well above the threshold for the positive control (*13*), suggesting that the T-SPOT.TB assay might be an appropriate test method for HIV-infected individuals with advanced immunodeficiency. However, previous studies suggest that advanced immunodeficiency may affect the performance of the T-SPOT.TB assay (*14-16*). The value of the T-SPOT.TB assay for the diagnosis of active TB in HIV-infected individuals with very low CD4+ T-cell counts has not yet been established.

The objectives of this retrospective study in HIV-infected individuals were to determine the performance of the T-SPOT.TB assay among active tuberculosis patients whose CD4+ T-cell counts were below 10 and 50 cells/μL. The performance of the T-SPOT.TB assay in patients with CD4+ T-cell counts below 10 cells/μL has not been reported.

2. Materials and Methods

2.1. *Selection of study subjects*

One hundred HIV-infected patients naïve to anti-TB therapies were enrolled from July 2012 to October 2013 at Shanghai Pubic Health Clinical Center in Shanghai, China. The patients diagnosed with confirmed active TB had presented to the clinic with signs and symptoms of TB. Written informed consent was obtained from all participants.

2.2. *Procedures*

All patients had a symptom assessment, a full clinical assessment, and sputum (three samples if productive or at least one sample induced with hypertonic 3% saline if not) was sent for smear microscopy and mycobacterial culturing. Chest radiographs and/or CT scans were performed. As needed, other appropriate specimens were obtained for microscopy and culturing. A fixed number of peripheral blood mononuclear cells from venous blood samples were used to perform the T-SPOT.TB assay according to the manufacturer's instructions using the T-SPOT.TB kit (Oxford Immunotec Ltd., Oxford, UK). Test wells were considered positive if the number of spot-forming cells obtained from the test antigens, either or both of the ESAT-6 or CFP-10-derived peptides, was more than twice that of the negative control, and if they had six or more spots than the negative control. Wherever possible, patients with indeterminate results had another sample tested until a definitive positive or negative result was achieved.

2.3. *Statistical methods*

Chi-squared (χ^2) tests were used for proportional comparisons among different subgroups. The Wilcoxon rank-sum test was used for non-normal data. The Shapiro-Wilk test was used to assess normality of the data. Spearman's rank correlation was used for correlation calculations. All *p*-values were two-sided with $\alpha = 0.05$. All data were analyzed using STATA 10.0 software (StataCorp, College Station, Texas, USA).

3. Results

3.1. *General information of the patients studied*

The average age of the patients was 42.0 ± 13.8 years. Eighty-nine percent of the cohort was male. 45% patients had specimens (sputum, stool, or abdominal cavity effusion) that were smear microscopy positive. For other characteristics see Tables 1 and 2.

3.2. *Analysis of positive and negative T-SPOT.TB performance results*

The sensitivity of the T-SPOT.TB assay was 59% among the 100 patients. Patients with lower CD4+ T-cell counts (11-50 cells/μL) had the lowest T-SPOT.TB positive rates (50%). Patients with higher CD4+ T-cell counts (50-100 cells/μL) had the highest T-SPOT.TB positive rates (75%). There were no significant differences between the T-SPOT.TB positive rates of patients with different CD4+ T-cell counts ($p = 0.287$). We divided the CD4 + T-cell counts into two categories, 1-50 cells/μL and > 50 cells/μL, although there was still no significant association ($p = 0.082$).

The patients with positive TB culture results had significantly higher T-SPOT.TB positive rates (78.9%) than the TB culture-negative group (44.3%) ($p < 0.001$). Other variables, including gender, age, TB disease classification, HIV RNA level, and highly reactive antiretroviral therapy (HAART), had no significant effects on T-SPOT.TB positive rates (Table 2).

Table 1. Group and subgroup of patients with active tuberculosis

Group	Number	Subgroup	Number
Pulmonary TB	59	Ordinary pulmonary TB	57
		Blood disseminated pulmonary TB	2
Extrapulmonary TB	15	Lymph node TB	5
		Tuberculous meningitis	5
		Pancreatic TB	1
		Tuberculous peritonitis TB	1
		Tuberculous pleurisy and spinal TB	1
		Tuberculous meningitis and lymph node TB	1
		Intestinal and abdominal cavity lymph node TB	1
Pulmonary and extrapulmonary TB	26	Pulmonary TB and tuberculous pleurisy	8
		Pulmonary TB and tuberculous meningitis	5
		Pulmonary TB and lymph node TB	4
		Pulmonary and intestinal TB	2
		Pulmonary and laryngeal TB	1
		Pulmonary and mediastinal lymph node TB	1
		Pulmonary TB plus tuberculous meningitis and lymph node TB	1
		Pulmonary TB plus tuberculous pleurisy and lymph node TB	1
		Pulmonary TB plus tuberculous pleurisy and tuberculous peritonitis	1
		Blood disseminated pulmonary TB plus tuberculous pleurisy and lymph node TB	1
		Pulmonary TB, tuberculous pleurisy plus tuberculous peritonitis and lymph node TB	1

Table 2. General characteristics of the study subjects (n = 100) according to the results of the T-SPOT.TB assay

Variable	Positive T-SPOT.TB	Negative T-SPOT.TB	p-value
Subjects (n)	59	41	-
Gender			
Males	54	35	0.333
Females	5	6	
Age			
≤ 40 years	27	24	0.209
> 40 years	32	17	
TB disease			
Pulmonary	35	24	0.186
Extrapulmonary	6	9	
Pulmonary and extrapulmonary	18	8	
CD4+ T cell counts			
1-10 cells/μL	11 (52.4%)	10 (47.6%)	0.287
11-50 cells/μL	19 (50%)	19 (50%)	
51-100 cells/μL	15 (75%)	5 (25%)	
101-536 cells/μL	13 (61.9%)	8 (38.1%)	
1-50 cells/μL	30 (50.8%)	29 (49.2%)	0.082
51-536 cells/μL	28 (68.3%)	13 (31.7%)	
HIV RNA[a]			
≥40 copies/mL	38	32	0.482
<40 copies/mL	6	3	
HAART treatment			
YES	14 (63.6%)	8 (36.4%)	0.544
NO	44 (56.4%)	34 (43.6%)	
TB culture[aa]			
Positive	30 (78.9%)	8 (21.1%)	< 0.001
Negative	27 (44.3%)	34 (55.7%)	

[a] The HIV RNA of 21 patients was not detected. [aa] The culture results in two patients were other microorganisms.

3.3. *Correlation of CD4+ T-cells with T-SPOT.TB*

The number of spot-forming cells (SFCs) reactive with ESAT-6, CFP-10 and ESAT-6/CFP-10-specific T cells detected by T-SPOT.TB were positively correlated with the number of circulating CD4+ T-cells (Table 3).

4. Discussion

It is more difficult to detect tuberculosis infections in HIV-infected patients than in uninfected individuals due to the TB-associated decline of some immune phenomena that is secondary to the destruction of the immune system (*17*).

Table 3. Correlation of numbers of circulating CD4+ T-cells with numbers of SFCs in response to incubation with ESAT-6 and CFP-10 in the T-SPOT.TB test

Correlation of CD4+ T-cells per μL with	Spearman's rho	*p*-value
T-SPOT.TB ESAT-6 SFCs per million PBMC	0.3791	0.0001
T-SPOT.TB CFP-10 SFCs per million PBMC	0.2929	0.0031
T-SPOT.TB ESAT-6/CFP-10 SFCs per million PBMC	0.3345	0.0007

The T-SPOT.TB assay has been evaluated for the detection of TB in HIV-infected people by many studies, although there were only limited data from a small number of HIV-infected patients with advanced immunodeficiency (*17-19*), and there were few data regarding its performance in active TB patients with HIV and CD4+ T-cell counts below either 10 or 50 cells/μL.

Therefore, we examined the relationship between T-SPOT.TB responses and CD4+ T-cells in HIV infected patients with active tuberculosis. The sensitivity of the T-SPOT.TB assay was 59% in our study. This was greater than the reported sensitivity of the T-SPOT.TB test in HIV-1 infected persons with active TB in the studies of Chen *et al*. (*20*) and Yu *et al*. (*21*) (34.2%, 13/38; 41.3%, 19/46, respectively), poorer than the sensitivities reported in the studies of Clark *et al*. (*19*), Chen *et al*. (*22*) and Ling *et al*. (*23*) (90.3%, 28/30; 77.4%, 493/637; 82%, 80/98, respectively). A similar sensitivity was reported in the studies of Santin *et al*. (*24*), Metcalfe *et al*. (*25*), Oni *et al*. (*26*), Markova *et al*. (*27*), and Jiang *et al*. (*28*) (65%, 202/311; 68%, 177/261; 68%, 58/85; 62%, 8/12; 65.6%, 21/32, respectively). We speculate on the sensitivity of the T-SPOT.TB test in HIV-1 infected persons with active TB is approximately 70% in studies using a large number of samples; different results can be obtained from studies of small numbers of samples.

In our study, the groups of CD4+ T-cell counts were < 10, 11-50, 51-100 and > 100 cells/μL. The CD4+ T-cell counts in the first two groups were extremely low, especially in the first group. However, there were no significant differences among the T-SPOT. TB positive rates of patients with different CD4+ T-cell counts. The non-significant association between CD4+ T-cell counts and T-SPOT.TB responses might be due to the small number of subjects in each group of CD4+ T-cell counts. We subsequently divided the CD4+ T-cell counts into two categories, 1-50 and > 50 cells/μL. Nevertheless, there was still no statistically significant difference in T-SPOT.TB positive rates. Our results strongly support previous studies that showed T-SPOT.TB positive rates are not correlated with T-cell stratification in patients with HIV-infection and active TB (*20,26*). Chen *et al*. (*20*) divided the CD4+ T-cell counts into two categories, 1-99 and ≥ 100 cells/μL. Oni *et al*. (*26*) divided the CD4+ T-cell counts into four categories, < 100, 100-199, 200-299, and > 300 cells/μL. Neither study found a significant association between CD4+ T-cell counts and T-SPOT.TB responses. Consequently, we demonstrated that T-SPOT.TB positive results were not dependent on CD4+ T-cell counts in HIV-infected patients with active TB.

We found that the number of SFCs reactive with ESAT-6 and CFP-10 positively correlated with the number of circulating CD4+ T-cells. This result differs from those of previous studies (*8,9,20*). Chen *et al*. (*20*) did not find a correlation between CD4+ T-cell counts and the amount of SFCs reactive with ESAT-6 or CFP10 in 35 individuals with active TB and HIV infection. Leidl *et al*. (*9*), using the QFT-IT assay, found that in HIV-infected patients, the concentration of IFN-γ in response to specific M. tuberculosis antigens directly correlated with the number of circulating of CD4+ T-cells. In contrast, SFCs in the ESAT-6 or CFP-10 antigen wells in the T-SPOT.TB assay were not correlated to the numbers of circulating CD4+ T cells (*9*). In another study by Karam *et al*. (*8*), the total number of SFCs reactive to ESAT-6 and CFP10 did not vary with changes in CD4+ T-cell counts. However, the proportion of positive responses to the ELISPOT assay decreased with decreasing CD4+ T-cell counts in HIV-infected patients (*8*). The reasons that these results differ from those in our study might be because our study subjects were all active TB patients with HIV infections.

In our study, the T-SPOT.TB positive results in the group with positive TB cultures was significantly higher than the culture-negative group (78.9% *vs*. 44.3%, respectively). Our result is similar to previous observations that showed 75% T-SPOT.TB positive results in HIV infected TB patients with positive TB cultures, greater than 22% positive results for the culture negative group (*29*). Oni *et al*. (*26*) found 68% T-SPOT.TB positive results in 85 HIV infected TB patients with positive TB cultures. This result was slightly lower than ours. Ribeiro *et al*. (*30*) found the mean ESAT-6 or CFP-10 SFC counts were higher for the positive sputum culture group compared to the culture-negative group, although this difference did not reach statistical significance before patients received anti-TB treatment. The study individuals were composed of 58 patients with pulmonary tuberculosis, of whom 57 were HIV seronegative (*30*). Ling *et al*. (*31*) thought the T-SPOT.TB assay did not have added value beyond clinical data and conventional tests for diagnosis of TB in smear-negative children in a high-burden setting. Although the T-SPOT.TB positive rate

may be lower in HIV infected TB patients with negative TB cultures, TB cultures are time-consuming and the T-SPOT.TB assay is faster.

In conclusion, the SFCs in the T-SPOT.TB assay are strongly related to the degree of immunodeficiency, while the T-SPOT.TB positive rates are less dependent on the level of CD4+ T-cell depletion.

Our study has several limitations. First, we did not explain why the T-SPOT.TB positive results of patients with < 10 CD4+ T-cells/μL were similar to other groups that had greater CD4+ T-cell counts. Second, we collected a limited number (100) of active tuberculosis patients that were co-infected with HIV. Third, most of the individuals were male patients, which may have affected the results.

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Original Article

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Repeated doses of intravenous tranexamic acid are effective and safe at reducing perioperative blood loss in total knee arthroplasty

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Summary **Fibrin sealant (FS) and tranexamic acid (TXA) have been used in total knee arthroplasty (TKA) to minimize perioperative blood loss. The efficacy of FS has been debated, and few studies have looked into the effects of FS and TXA on perioperative coagulability. The current study retrospectively reviewed 100 cases of unilateral primary TKA. Twenty-five cases served as blank controls, FS was used without TXA in 23, TXA was used without FS in 20, and both FS and TXA (FS + TXA) were used in 32. FS was sprayed before wound closure whereas 1 g of TXA was intravenously administered before incision and 1 g was administered 15 min before tourniquet release. Hematocrit and hemoglobin levels and thromboelastography (TEG) parameters were assessed pre-operatively and on day 1, 4, and 9 post-operatively. Blood transfusions were noted and the incidence of symptomatic DVT/PE was determined. Hematocrit and hemoglobin levels were significantly higher in the TXA and FS + TXA groups compared to the control and FS groups on day 1, 4, and 9 post-operatively. Hematocrit and hemoglobin levels in the control group were similar to those in the FS group and hematocrit and hemoglobin levels in the TXA group were similar to those in the FS + TXA group. TEG parameters (R, K, α, MA, and CI) remained within normal ranges. Mean CI was less than +3 in all four groups, suggesting that hypercoagulation was not promoted. One patient in the FS group received an allogeneic transfusion. Incidence of symptomatic DVT/PE was not noted. Intravenous TXA significantly reduced perioperative blood loss in patients undergoing a TKA but FS did not. Administration of FS in addition to TXA was not superior to TXA alone. FS and/or TXA did not increase the risk of hypercoagulation according to TEG parameters. Intravenous administration of 1 g of TXA pre-operatively and administration of 1 g before tourniquet release is an effective and safe method of reducing blood loss in TKA.**

Keywords: Tranexamic acid, total knee arthroplasty, thromboelastography, fibrin sealant, blood loss

1. Introduction

Total knee arthroplasty (TKA) is considered one of the most successful orthopedic procedures as indicated by a high level of patient satisfaction. However, management of perioperative blood loss continues to be a significant concern and challenge. In addition to intraoperative loss and drain output, up to 50% of total blood loss in TKA consists of hidden blood loss such as residual hemarthrosis, extravasation of blood into surrounding tissues, and hemolysis (*1*). The total blood loss associated with TKA can be up to 1,700 mL (*1*), leading to a substantial risk of allogeneic blood transfusions, which in turn have the potential to lead to transfusion reactions, graft-versus-host disease, hyperkalemia, fluid overload, and infections (*2,3*).

Numerous alternatives have been used to avoid allogeneic blood transfusions, including preoperative autologous blood donation, epoetin alpha, iron supplements, normovolemic hemodilution, intraoperative blood cell salvaging, hypotensive

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anesthesia, and hemostatic agents (*4-7*), although these alternatives also have significant risks. For instance, retransfused blood from blood salvage systems can be hemolyzed and contain inflammatory mediators (*8*) and hypotensive anesthesia and normovolemic dilution can increase the risk of myocardial infarction, cerebral ischemic events, and even death (*7*).

Fibrin sealants (FS) and tranexamic acid (TXA) are two hemostatic agents widely used to reduce perioperative blood loss in TKA. FS achieves hemostasis by mimicking the final phase of the coagulation cascade in which thrombin converts fibrinogen into fibrin threads (*9*). TXA is an antifibrinolytic that binds to specific sites of both plasminogen and plasmin, competitively inhibiting the activation of plasminogen to plasmin and thus inhibiting dissolution of clots. Opinions are mixed over the true efficacy of FS in reducing perioperative blood loss and reducing the need for blood transfusion (*10-16*). In contrast, multiple trials have clearly demonstrated that TXA is highly reliable (*17-20*). However, few studies have compared the efficacy of FS and TXA or whether combined use of FS and TXA would be more effective than use of either alone since their mechanisms of action affect different stages of the coagulation pathway.

One of the major concerns for hemostatic agents, especially when they are systematically administered, is the increased risk of deep vein thrombosis (DVT) and pulmonary embolism (PE). Although most trials have not found FS and/or TXA to promote hypercoagulation after TKA, that possibility cannot be ruled out.

Conventional coagulation assays are performed only on plasma rather than on whole blood. Therefore, the role of platelets in clot strength and formation is often not considered. Thromboelastography (TEG) is a method of testing the efficiency of blood coagulation, including parameters that conventional assays cannot measure, *e.g.*, platelet function, clot strength, and fibrinolysis. The current study attempted to compare combined and individual use of FS and TXA in order to provide additional evidence of their efficacy in reducing perioperative blood loss during TKA. This study also sought to use TEG to determine whether these agents would promote hypercoagulation in patients undergoing TKA.

2. Materials and Methods

2.1. *Patient characteristics*

Cases of patients who suffered from osteoarthritis or rheumatoid arthritis and who underwent selective unilateral TKA at this Hospital from January 2012 to April 2014 were retrospectively reviewed. Patients with a physical status of I-III according to the American Society of Anesthesiologists (ASA) were enrolled in the study. Patients with history of thromboembolic disease, recent anti-coagulation therapy, chronic renal failure, or anemia (preoperative Hb < 110 g/L) and those who were allergic to FS or TXA were excluded. This study was approved by the ethics committee of this Hospital.

2.2. *Surgical and perioperative treatment*

A medial parapatellar approach and pneumatic tourniquet were used for all surgeries. Cemented prostheses (Genesis II, Smith & Nephew, Memphis, Tennessee, USA and Palacos R+G, Heraeus Medical, Wehrheim, Germany) were implanted in all patients. Before cementing, an autologous bone plug was used to fill the hole in the femoral canal left by the intramedullary guide. Local infiltration anesthesia was given after successful prosthesis implantation. Bleeding was precisely controlled with electrocautery. For topical application of FS, 5 mL of fibrin sealant (human thrombin and fibrinogen, Shanghai RAAS, Shanghai, China) was sprayed over the surgical site before wound closure. For intravenous administration of TXA (Jinhua Conba, Jinhua, Zhejiang Province, China) (IV-TXA), 1 g was administered before incision and 1 g was administered 15 min before tourniquet release. A single vacuum drain was used in the first 24 h after TKA and retrieved on the morning of day 2 post-operatively. Low molecular weight heparin (LMWH, Fraxiparine, GlaxoSmithKline, Notre-Dame-de-Bondeville, France) was administered immediately for thromboembolic prophylaxis and was subsequently administered daily until day 14 post-operatively. All surgeries were performed after general anesthesia with a femoral nerve block. An allogeneic blood transfusion was deemed necessary if Hb < 80 g/L during surgery.

2.3. *Data collection*

Hematocrit (Hct) and hemoglobin (Hb) levels and TEG parameters (R, K, α, MA, and CI) were assessed pre-operatively and on day 1, 4, and 9 post-operatively. The incidence of symptomatic DVT/PE was determined and diagnosed with ultrasound and CTA during hospitalization. Transfusion details were also recorded.
TEG was performed with kaolin as a coagulation activator and was measured with a TEG5000 hemostasis analyzer (Haemoscope, Niles, Illinois, USA). Numerous parameters indicating clot formation are determined by TEG. R is the time elapsed until the first evidence of a clot. K is the time from the end of R until the clot reaches 20 mm and represents the speed of clot formation. α is the angle of the curve made as K is reached and offers information like that from K. The maximum amplitude (MA) is an indication of the maximum strength of the clot. A mathematical formula can be used to determine a coagulation index (CI) (or overall assessment of coagulability) that

takes of these parameters into account (21). In TEG, hypercoagulability can be defined as CI > +3 (22).

2.4. *Statistical analysis*

Statistical data were presented as means ± standard deviation (S.D.) for continuous variables with a normal distribution and *n* (%) for categorical variables. One-way ANOVA was used to compare the means of continuous variables with a normal distribution and Pearson's Chi-square test was used to compare the proportion of categorical variables for the control, FS, TXA, and FS + TXA groups. All statistical analyses were performed using SPSS19.0 (IBM, Armonk, New York, USA). A $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. *Patient selection*

A total of 100 patients met the aforementioned criteria. No hemostatic agents had been used in 25 of these patients (control, group 1, *n1* = 25), topical FS spray but not TXA had been used in 23 patients (FS, group 2, *n2* = 23), intravenous TXA but not FS had been used in 20 patients (TXA, group 3, *n3* = 20), and both FS and TXA had been used in 32 patients (FS + TXA, group 4, *n4* = 32). There were no significant differences in patient demographics, including gender, age, and body weight (Table 1).

3.2. *Comparisons of peri-operative blood loss*

Preoperative Hct and Hb levels were comparable in the four groups. Blood loss was significantly reduced on day 1, 4, and 9 post-operatively as Hct and Hb levels were considerably higher in the TXA and FS + TXA groups (Table 2, Figure 1). There were no differences between the control group and FS group and between the TXA group and FS + TXA group. Hct and Hb levels were significantly higher in the TXA group than in the FS group (Hct: day 4, Hb: day 1 and 4).

3.3. *Comparison of the change in coagulation*

TEG parameters (R, K, α, MA, and CI) were comparable preoperatively. There were no significant differences in R, K, α, MA, or CI on day 1, 4, and 9 post-operatively, with the exception of K that differed slightly in the four groups on day 1 (control *vs*. FS, $p = 0.023$; TXA *vs*. FS + TXA, $p = 0.046$). Despite these differences, the means of TEG parameters remained within their normal ranges during the assessed perioperative period (Table 3 and Figure 2).

3.4. *Transfusions and DVT/PE*

Table 1. Patient characteristics preoperatively

Items	Group 1 (Control) *n1* = 25	Group 2 (FS) *n2* = 23	Group 3 (TXA) *n3* = 20	Group 4 (FS+TXA) *n4* = 32	Total *n* = 100	*p*
Gender, n(%)						0.181
Male	7 (28.0)	2 (8.7)	5 (25.0)	11 (34.4)	25 (25.0)	
Female	18 (72.0)	21 (91.3)	15 (75.0)	21 (65.6)	75 (75.0)	
Age (years)						0.420
Mean ± S.D.	71.52 ± 8.18	68.30 ± 7.49	70.80 ± 6.24	69.78 ± 5.83	70.08 ± 6.94	
Min, Max	56, 87	54, 83	61, 83	56, 80	54, 87	
Weight (kg)						0.316
Mean ± S.D.	67.48 ± 8.81	65.96 ± 13.15	70.85 ± 10.86	70.25 ± 8.54	68.69 ± 10.32	
Min, Max	46, 88	43, 95	55, 90	53, 91	43, 95	

Table 2. RBC and Hb on day 1, 4, and 9 post-operatively

Items	Group 1 (Control) *n1* = 25	Group 2 (FS) *n2* = 23	Group 3 (TXA) *n3* = 20	Group 4 (FS+TXA) *n4* = 32	*p*
Preoperatively					
Hct (%)	38.69 ± 2 .49	39.13 ± 3.45	38.80 ± 2.30	39.20 ± 3.49	0.912
Hb (g/l)	126.8 ± 10.9	128.1 ± 12.2	129.0 ± 8.0	131.2 ± 12.2	0.507
Day 1					
Hct (%)	30.02 ± 6.71	31.88 ± 3.55	33.59 ± 2.40	34.58 ± 2.94	0.001
Hb (g/l)	102.8 ± 11.4	105.3 ± 13.0	112.5 ± 7.8	116.7 ± 10.2	0.000
Day 4					
Hct (%)	28.63 ± 3.83	28.65 ± 3.42	31.13 ± 2.84	31.44 ± 3.89	0.004
Hb (g/l)	93.9 ± 14.6	95.4 ± 12.9	104.2 ± 8.7	106.3 ± 13.4	0.001
Day 9					
Hct (%)	29.96 ± 3.19	30.93 ± 3.64	32.43 ± 2.28	32.11 ± 3.08	0.025
Hb (g/l)	97.6 ± 11.6	101.1 ± 13.3	106.8 ± 7.4	103.3 ± 11.8	0.006

One patient in the FS group received 2 units of RBC and 200 mL of plasma. No incidence of symptomatic DVT/PE was observed in any of the 100 patients.

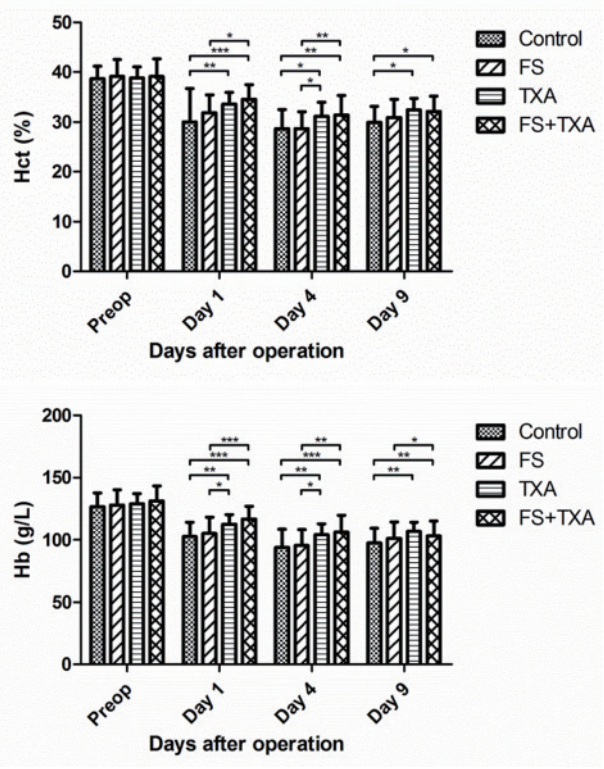

Figure 1. Perioperative blood loss in TKA. Groups that received TXA (TXA, FS + TXA) had significantly higher Hct and Hb levels postoperatively than those that did not (control/FS). **(a)** Hematocrit. **(b)** Hemoglobin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

As shown in this study, groups given TXA (TXA and FS + TXA) had higher Hct and Hb levels during the postoperative period while FS alone did not conspicuously reduce postoperative blood loss. Prior to this study, Aguilera *et al*. arrived at a similar conclusion, favoring IV-TXA over FS (*15*). The efficacy of FS has been debated as some surgeons have found FS to be ineffective (*14-16*). Their finding could be true or it could also be due to insufficient dosage (*23*) or improper topical application (*24*). In addition, certain FS formulations contain TXA, *e.g*. Quixil (Ethicon, Johnson & Johnson) (*25*). The current study used 5 mL of FS based on the contention by Notarnicola *et al*. that the efficacy of 5 mL of FS was comparable to a larger dose (*26*). However, the current findings suggest that FS was ineffective, regardless of whether it was administered in combination or individually.

Although the current data have further corroborated the efficacy of IV-TXA, there is still debate over the method of delivery, optimal dosage, and timing and duration of TXA administration. Zohar *et al*. (*27*) evaluated a 1-g oral dose (O-TXA) preoperatively and 3 1-g doses every 6 h postoperatively and found O-TXA to have comparable efficacy to IV-TXA, although O-TXA was more convenient. Irwin *et al*. favored O-TXA because it was more cost-effective than IV-TXA (28). Wong *et al*. (*29*) reduced postoperative bleeding by 20%-25% with intra-articular TXA injections (IA-TXA) after wound closure. IA-TXA was proven effective for both cemented and cementless

Table 3. Thromboelastography on day 1, 4, and 9 post-operatively

Items	Group 1 (Control) *n1* = 25	Group 2 (FS) *n2* = 23	Group 3 (TXA) *n3* = 20	Group 4 (FS+TXA) *n4* = 32	*p*
Preoperatively					
R (min)	6.00 ± 0.90	5.86 ± 0.90	5.95 ± 0.79	5.43 ± 0.95	0.062
K (min)	1.79 ± 0.38	1.60 ± 0.25	1.64 ± 0.30	1.86 ± 0.66	0.315
α (°)	69.93 ± 3.95	72.30 ± 2.40	71.76 ± 2.82	70.88 ± 5.67	0.128
MA (mm)	63.75 ± 4.67	64.47 ± 3.37	62.52 ± 4.68	62.34 ± 5.96	0.361
CI	0.74 ± 1.32	1.16 ± 0.99	0.82 ± 1.13	0.99 ± 1.65	0.712
Day 1					
R (min)	5.02 ± 1.02	4.92 ± 1.03	4.86 ± 0.83	5.10 ± 0.92	0.798
K (min)	1.59 ± 0.44	1.36 ± 0.25	1.38 ± 0.26	1.68 ± 0.52	0.315
α (°)	72.14 ± 4.65	74.64 ± 2.40	74.37 ± 2.40	72.01 ± 4.73	0.128
MA (mm)	63.63 ± 4.69	64.82 ± 4.20	64.33 ± 4.66	64.35 ± 5.07	0.852
CI	1.60 ± 1.48	2.08 ± 1.06	2.03 ± 1.21	1.59 ± 1.35	0.372
Day 4					
R (min)	5.36 ± 0.96	5.52 ± 0.92	5.12 ± 0.74	4.93 ± 1.04	0.109
K (min)	1.39 ± 0.29	1.35 ± 0.36	1.22 ± 0.29	1.30 ± 0.31	0.304
α (°)	74.04 ± 2.98	75.07 ± 3.02	76.13 ± 2.35	75.11 ± 2.98	0.125
MA (mm)	67.96 ± 4.67	65.71 ± 6.62	67.30 ± 5.53	67.37 ± 4.10	0.489
CI	2.13 ± 1.08	1.80 ± 1.10	2.42 ± 1.13	2.33 ± 1.29	0.287
Day 9					
R (min)	5.30 ± 1.24	5.55 ± 0.77	5.69 ± 1.12	5.39 ± 0.90	0.572
K (min)	1.19 ± 0.27	1.13 ± 0.17	1.18 ± 0.28	1.28 ± 0.40	0.329
α (°)	76.32 ± 2.92	76.90 ± 1.87	76.65 ± 2.87	75.58 ± 4.23	0.456
MA (mm)	70.64 ± 3.83	71.32 ± 3.15	69.94 ± 5.47	71.20 ± 4.53	0.699
CI	2.75 ± 1.31	2.71 ± 0.89	2.44 ± 1.26	2.65 ± 1.33	0.846

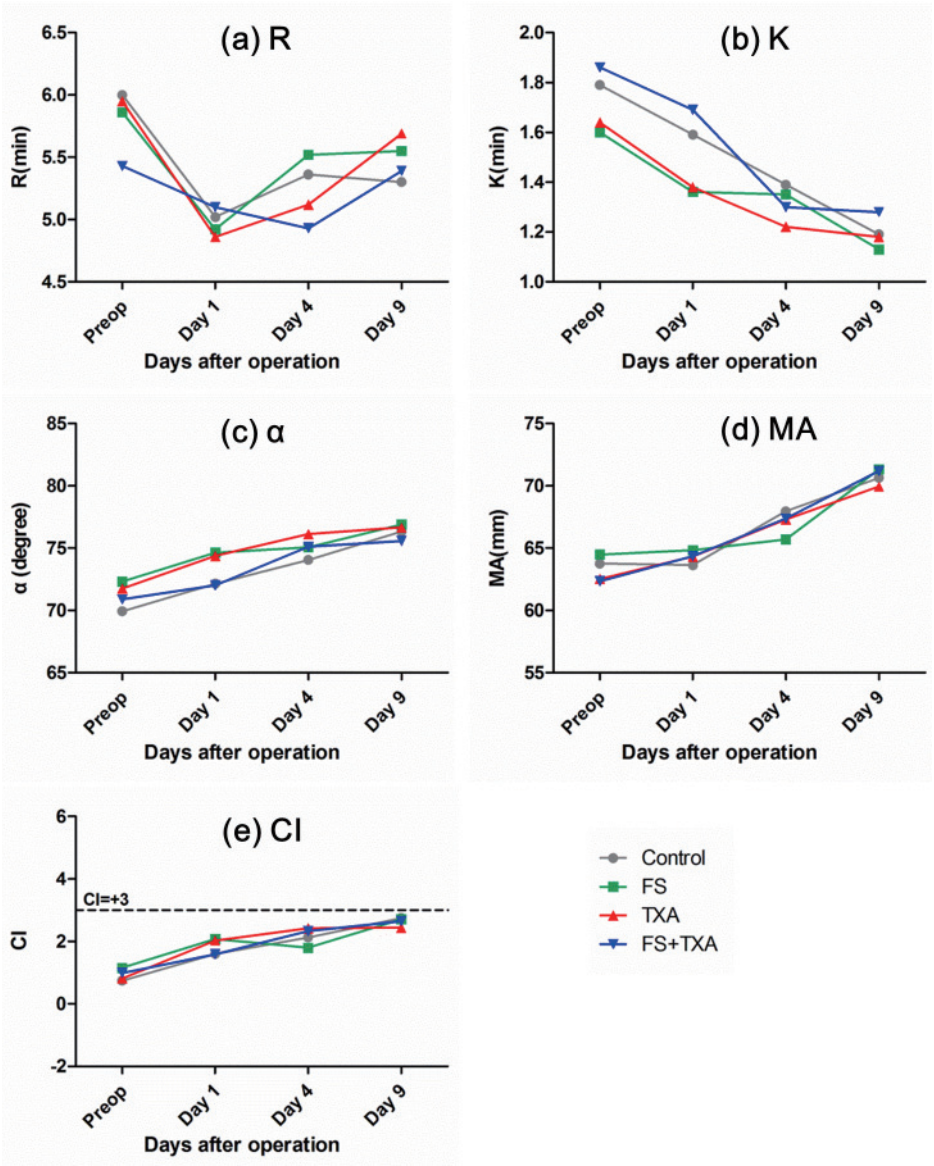

Figure 2. Thromboelastography on day 1, 4, and 9 post-operatively. There were no significant differences in R, K, α, MA, or CI on day 1, 4, and 9 post-operatively. Means of all of the parameters remained within their normal ranges during the assessed perioperative period. Mean CI was less than +3 in all groups, suggesting that neither FS nor TXA promoted hypercoagulation.

implants (*30-33*) and favored based on the concern that systematic administration of IV-TXA might potentially increase the risk of thrombosis (*34*). In contrast, the TEG data obtained in the current study suggest that IV-TXA did not promote hypercoagulation since the mean CI in the TXA and FS + TXA groups was less than +3 and this CI remained similar to that of the control group during hospitalization. Thus, one could infer that IV-TXA does not increase the risk of developing DVT.

Like this study, most trials favor IV-TXA and have not noted an increased onset of clinically significant thrombosis. A recent meta-analysis reviewed 19 randomized controlled trials (RCTs) and concluded that a multiple-dose regimen and total dose ≥ 30 mg/kg IV-TXA significantly reduced postoperative blood loss by a mean of 290 mL and total blood loss by a mean of 570 mL in comparison to a saline control (*35*). Most RCTs have found no significant reduction in intra-operative blood loss between TXA and control groups (*35*). Tanaka *et al*. (*19*) posited that TXA would have its greatest hemostatic effect once before surgery and once before release of the tourniquet because suppression of fibrinolysis at the start of surgery may be more effective than suppression only when hyperfibrinolysis peaks.

Due to the scarcity of an allogeneic blood supply at this Hospital, a strict criterion for transfusion was in effect in the form of intraoperative Hb < 80 g/L. Only one patient in the FS group required such a transfusion in accordance with this protocol. None of the other patients received blood transfusions of any kind. Results were limited, so the reduction in the number of blood transfusions was not analyzed. In evidence provided by the literature (*35*), O-TXA, IA-TXA,

and IV-TXA all have the ability to reduce rates and quantities of blood transfusions in TKA. Like reduction of perioperative blood loss, whether FS can reduce the need for allogeneic transfusion is a question that has yet to be answered (*16,36*).

The current study included a few patients with coronary stents and/or brain infarctions who depended on regular anti-platelet treatment with aspirin and/or clopidogrel hydrogen and who stopped that medication one week prior to surgery. Caution was exercised with regard to whether IV-TXA would further disturb coagulation in these patients who were at risk for thromboembolic events. In a trial of high-risk patients who were ASA III-IV conducted by Whiting *et al*. (*37*), TXA was not associated with an increase in symptomatic thromboembolic events (6.7% *vs*. 4.3%; $p = 0.270$) and was associated with a decrease in transfusion rates (17% *vs*. 48%; $p = 0.001$). Similarly, the current findings revealed few differences in the TEG parameters of all four groups, suggesting that both TXA and FS slightly affect coagulation in patients with severe comorbidities.

In this retrospective study, FS and TXA were used either individually or in combination depending on various findings and conjectures concerning the use of hemostatic agents in the literature at the time. This led to an uneven distribution of patients in the four groups. A well-designed RCT would serve to refine the results of this investigation.

In summary, IV-TXA significantly reduced perioperative blood loss during TKA. FS did not significantly reduce blood loss and it was less effective than TXA. FS in addition to IV-TXA was not superior to IV-TXA alone. TEG parameters (R, K, α, MA, CI) were similar for all four groups, suggesting that neither FS nor IV-TXA increased the risk of hypercoagulation, regardless of whether the agent was administered individually or in combination. At this Hospital, topical FS is no longer in use but IV-TXA has been incorporated into the care protocol. One g of IV-TXA is administered pre-operatively and 1 g is administered before tourniquet release, which appears to be an effective and safe method of reducing perioperative blood loss in TKA.

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Original Article

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Effects of lifestyle factors on urinary oxidative stress and serum antioxidant markers in pregnant Japanese women: A cohort study

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Summary **Oxidative stress plays a major pathological role in pregnancy-related complications. Although oxidative stress is induced by exogenous toxins in association with a poor lifestyle in normal subjects, there is little information on the factors altering oxidative stress and antioxidant levels during pregnancy. The purpose of this study was to determine the relationship between lifestyle factors and oxidative stress/antioxidant levels during each trimester and 1-month postpartum. This prospective cohort study followed 54 healthy women through pregnancy; first, second, and third trimester and 1-month postpartum. Participants were administered a questionnaire on characteristics and lifestyle factors. Morning blood and urine samples were obtained to measure urinary biopyrrins and serum coenzyme Q10 (CoQ10) levels. The levels of urinary biopyrrins and serum CoQ10 increased significantly throughout pregnancy, with peak values registered during the third trimester. Higher biopyrrin levels were significantly associated with non-consumption of morning meal during the first trimester, smoking during the third trimester and 1-month postpartum, alcohol consumption during the third trimester, high food-based polyunsaturated fatty acid intake during the third trimester, and poor mental health scores during the first and third trimesters. Higher CoQ10 levels were significantly associated with no smoking during pregnancy and at 1-month postpartum, and with a high frequency of exercise during the third trimester and 1-month postpartum. Thus, pregnancy represents a state of oxidative stress, which can be counterbalanced by increased levels of antioxidants, such as CoQ10. We speculate that certain lifestyle choices such as avoiding smoking can reduce oxidative stress and increase antioxidant levels during pregnancy.**

Keywords: Biopyrrin, life style, oxidative stress marker, pregnant women, prenatal care

1. Introduction

Various complications of pregnancy, such as preeclampsia (PE), pregnancy-induced hypertension (PIH), and gestational diabetes mellitus (GDM), are associated with oxidative stress (*1-5*). Thus, clinical and experimental evidence indicates that oxidative stress is important in the aetiology of PE, PIH, and GDM. The levels of oxidative stress markers, such as oxidised low-density lipoprotein (LDL) and malondialdehyde, are higher (*6*) and those of antioxidants, such as coenzyme Q10 (CoQ10), α-tocopherol, and vitamin C, are lower (*1,3,7*) in women experiencing PE, PIH, or GDM, compared with healthy pregnant women. Accumulation

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of Reactive oxygen species (ROS) in cells may cause DNA damage (*8*), which may lead to pregnancy-related complications.

Increased oxidative stress and associated DNA damage can be induced by endogenous toxins released during inflammation and by exogenous toxins related to poor lifestyle, such as smoking (*9*), excessive drinking (*10*), and poor mental health (*e.g.*, psychological stress) (*11,12*). On the other hand, the DNA repair process is accelerated by various endogenous and exogenous factors, such as exercise and vitamins, in non-pregnant individuals (*13,14*). Previous studies have reported that the overall health of pregnant women and their infants is influenced by oxidative stress associated with lifestyle factors, such as smoking and vitamin intake (*15-17*). Aycicek and Ipek (*16*) noted high levels of the free radical lipid hydroperoxide (LOOH) and low levels of catalase and total antioxidant capacity (TAC) in the cord blood of pregnant smokers. They also reported a correspondingly higher incidence of low infant body weights in pregnant smokers than in non-smokers (*16*). Another study found that antioxidant supplementation in pregnant women with low levels of superoxide dismutase, an antioxidant, during the first trimester decreased the incidence of PE (*17*). However, there is little or no information on the lifestyle factors that alters oxidative stress during pregnancy. Therefore, the purpose of this study was to *i*) examine oxidative stress levels during normal pregnancy and at 1-month postpartum, and *ii*) identify the lifestyle factors that have an impact on oxidative stress and antioxidants levels at the same time points.

2. Materials and Methods

2.1. *Study design and enrolment*

This prospective cohort study was conducted at an obstetrics and gynaecology clinic in Tokyo between July 2004 and March 2005. Healthy women with singleton pregnancies, who were free of pregnancy complications and chronic illnesses, were asked to participate in this study; the women were followed individually, between the first trimester to 1-month postpartum. Sixty-two of the 75 (82.7%) pregnant women, at 12-13 weeks gestation, who visited the clinic for check-up between July and August 2004 agreed to participate in the study. Demographic data and the results for 8 of the 62 participants were accidentally lost during the study. Therefore, data collected during the first, second, and third trimesters, and at 1-month postpartum from 54 (72.0%) participants were available for analysis. Each woman underwent an ultrasonography examination, prior to enrolment, to determine the accurate gestational age. The study protocol was approved by the ethics review committee of the Graduate School of Medicine, The University of Tokyo, and written informed consent was obtained from each participant.

2.2. *Biological sample collection*

At their clinic visits during the first (12 weeks), second (22 weeks), and third (32 weeks) trimester and at 1-month postpartum, participants were asked to complete a food frequency questionnaire and a questionnaire on basic characteristics and lifestyle factors. Morning blood and urine samples were obtained at the time of routine check-ups at the same time when the questionnaires were completed.

After allowing the blood to clot at room temperature, the blood samples were centrifuged at 1,610× g for 10 min, and stored at -50°C. The urine samples were immediately protected from exposure to light and stored at -50°C. All samples were analysed within 6 months.

2.3. *Characteristics and lifestyle data*

Maternal age, gravidity, parity, pre-pregnancy body mass index (kg/m^2), blood pressure (mmHg), and urinary protein levels during pregnancy and at 1-month postpartum, together with data on the newborns, such as gestational weeks at delivery (weeks), birth weights (g), and placental weights (g) were collected from the maternal medical records. Lifestyle variables related to high and low ROS and antioxidant levels, such as current intake of vitamin supplements, alcohol consumption, smoking, and exercise habits, night time sleep (hours), and working status, were obtained from the questionnaire.

2.4. *Mental heath*

Mental health was evaluated by the 12-item General Health Questionnaire (GHQ-12) (*18-20*). The Cronbach's alpha for this study was 0.85. A high GHQ-12 score represents low mental health level. The cut-off point was set at a GHQ-12 score of 4 points (*21*).

2.5. *Dietary data*

The brief, self-administered, diet history questionnaire (BDHQ) was used to obtain data on the intake of various foods during the preceding month (*22*). The BDHQ included 74 questions on the frequency of consumption of 62 specified food items, seasonings, supplements, beverages, and others. Seven or eight frequency responses were used for ranking, from 'never' to 'more than 4 times per day'. Based on the type of consumed foods, the main nutrients consumed by each participant were selected based on the Standard Tables of Food Composition in Japan (5th ed.) (*23*).

2.6. *Measurements of urinary biopyrrin and serum CoQ10 levels*

Biopyrrins are oxidative metabolites of bilirubin and are generated under stress conditions such as poor mental health, infection, ischemia, and surgery (*11,24,25*). We have focused on the biopyrrin level in the clinical setting because it involves noninvasive sampling and reflects lifestyle factors such as psychological status (*11*). Therefore, biopyrrin was adopted as a marker for oxidative stress because it is a predictor of optimal lifestyle during pregnancy. Furthermore, the antioxidant CoQ10 was used to clarify the relationship between oxidative stress and pregnancy complications in previous studies (*1,3*). In addition, CoQ10 has a strong antioxidant effect on the human body; hence, it was considered as a marker for oxidative stress.

2.6.1. *Measurement of urinary biopyrrin*

The biopyrrin levels were measured in duplicate, using a biopyrrin enzyme immunoassay kit that employs an alkaline phosphatase-labelled 24G7 anti-bilirubin monoclonal antibody (Cat. No. B433, Shino-test Corp., Kanagawa, Japan) (*26,27*). The results were expressed relative to urinary creatinine concentrations, which were measured enzymatically (Mitsubishi Chemical Medience, Tokyo, Japan). The biopyrrin immunoassay using 24G7 monoclonal antibody is influenced by hyperbilirubinemia (*26,27*); therefore, the measured urinary biopyrrin level may be influenced by the serum direct bilirubin level. Therefore, the latter was measured by a chemical oxidation-based method (Mitsubishi Chemical Medience, Tokyo, Japan).

2.6.2. *Measurement of serum CoQ10*

Serum CoQ10 levels were measured by high-performance liquid chromatography (HPLC) with an electrochemical detector system (Nanospace S1-2, Shiseido Co. Ltd., Tokyo, Japan) according to the modified method used for the assay of saliva CoQ10 levels, reported by Sekine *et al*. (*28*). The standard CoQ10 sample was kindly provided by Kaneka Co. (Osaka, Japan) through Shiseido Co., Ltd. Other chemicals required for the measurement included HPLC-grade isopropanol (catalog #29128-31, Nacalai Tesque Inc., Kyoto, Japan), methanol (#21929-23, Nacalai Tesque Inc.), and sodium periodate (catalog #410241-100G, Sigma-Aldrich, St Louis, MO, USA). The HPLC system consisted of a concentration column (Capcell Pak C8, 4 × 10 mm, 5 μm), a reduction column (Shiseido CQ, 2 × 20 mm), a separation column (Capcell Pak C18 AQ, 2 × 75 mm, 3 μm), and an electrochemical detector (650 mV) (Nanospace S1-2 3016, Shiseido Co., Ltd). When the samples were loaded, a mobile phase of 50 mM $NaClO_4$ in methanol/distilled water (95/5, v/v) was used. Next, using a column-switching system, serum CoQ10 was eluted from the concentrating column by mobile phase 2 (50 mM NaClO4 in methanol/distilled water (95/5, v/v)). The column oven temperature was set at 40°C. The calibration range of the HPLC system was 1.57-200 ng/mL.

2.7. *Statistical analysis*

Data are expressed as means ± S.D. The generalised linear mixed model, χ^2 test, or Exact test was used for the analysis of lifestyle factors. The relationship between lifestyle factors and biopyrrin or CoQ10 levels at each trimester and 1-month postpartum was tested by multiple regression analyses. The independent variables with clinically relevant factors were selected for multivariate regression analyses. The levels of urinary biopyrrins and serum CoQ10 at the 4 assessment points were tested using a generalised linear mixed model, with the time during pregnancy as the fixed effect and the individuals as random effects. Bonferroni's post-hoc comparisons were used to identify significant differences among the 4 assessment time points. A p-value < 0.05 (two-sided test) was considered significant. All data were analyzed using the Statistical Package for Social Sciences for Windows, version 18.0 (SPSS Japan Inc., Tokyo, Japan).

3. Results

3.1. *Participants*

Table 1 summarises the characteristics and pregnancy outcomes of the participants. The mean age of the 54 women was 31.0 years; 19 women (35.2%) were primigravida, and none developed complications during pregnancy. Although delivery occurred at full-term in all women (between 37 and 42 weeks), 3 newborns (5.6%) were of low birth weight (< 2,500 g), whereas one (1.9%) experienced macrosomia (≥ 4,000 g).

Table 1. Characteristics of pregnant women (n = 54)

Age (years)[1)]	31.0 (22.0 - 39.0)
Parity	1.0 (0 - 4.0)
0	19 (35.2)
1	27 (50.0)
2	7 (13.0)
3	0
4	1 (1.8)
Pre-pregnancy BMI (kg/m^2)[2)]	20.3 (16.1 - 34.7)
Blood pressure ≥ 140/90mmHg[3)]	0
Urinary Protein ≥ 1+[3,4)]	14 (25.9)
Newborn	
Gestational week at delivery (weeks)	39.0 (37.0 - 41.0)
Birth weight (g)	3092.5 (2290.0 - 4300.0)
Low birth weight: < 2500 (g)	3 (5.6)
High birth weight: ≥ 4000 (g)	1 (1.9)
Placenta weight (g)	570.0 (370.0 - 810.0)

Data are median (range; minimum - maximum) or n (%). [1)] Age at study entry. [2)] Body Mass Index measured before a pregnancy. [3)] Data obtained during pregnancy and at one month postpartum.[4)] Dipstick with trace protein was used to detect urine protein. Protein 1+: 30 mg/dl.

3.2. *Biomarker levels*

The mean serum direct bilirubin level was stable during the study (first trimester: 0.24 mg/dL, second trimester: 0.15 mg/dL, third trimester: 0.24 mg/dL, 1-month postpartum: 0.16 mg/dL). The mean urinary biopyrrin level during the first trimester was 2.90 μmol/g Cre, but subsequently increased to 4.95 μmol/g Cre at the second trimester ($p < 0.001$), with a further increase of 1.3-fold in the third trimester (6.27 μmol/g Cre, $p = 0.004$); however, the mean urinary biopyrrin level decreased at 1-month postpartum to 3.52 μmol/g Cre, relative to the level at the third trimester ($p < 0.001$) (Figure 1). Further comparisons showed that the mean urinary biopyrrin level during the third trimester was significantly higher than that during the first trimester ($p < 0.001$), but there was no significant difference between the levels during the first trimester and 1-month postpartum ($p = 0.569$).

Figure 2 depicts serum CoQ10 levels during pregnancy and after delivery. The mean CoQ10 level increased significantly from 631 ng/mL during the first trimester, to 1,080 ng/mL during the second trimester ($p < 0.001$), and further to 1,639 ng/mL during the third trimester ($p < 0.001$); however, the mean CoQ10 level decreased after delivery to 919 ng/mL, relative to the level at the third trimester ($p < 0.001$). Further comparisons indicated a significant difference between the levels during the first and third trimesters ($p < 0.001$) and between the levels during the first trimester and 1-month postpartum ($p < 0.001$).

3.3. *Relationship between urinary biopyrrin and serum CoQ10 levels*

Spearman's correlation analysis between urinary biopyrrin and serum CoQ10 levels was performed after adjusting for the serum direct bilirubin level and the mean age during each trimester and 1-month postpartum. The Spearman's correlation coefficients were –0.04 for the first trimester and –0.21 for the third trimester (no significant difference), and –0.32 for the second trimester and –0.31 for 1-month postpartum (both, $p < 0.05$).

In addition, multiple linear regression analysis was performed to examine the relationship between the area under the curve (AUC) of the urinary biopyrrin and serum CoQ10 levels measured during the first, second, and third trimesters, and 1-month postpartum, after adjusting for age and serum direct bilirubin levels measured at the same time points. The standardised partial regression coefficient (β) of the relationship was –0.303 ($p < 0.001$), indicating a significant negative correlation between the levels of the 2 indicators. The goodness of fit of the statistical model (adjusted R^2) was 0.33 ($p < 0.001$).

3.4. *Relationship between oxidative stress, antioxidants, and lifestyle factors*

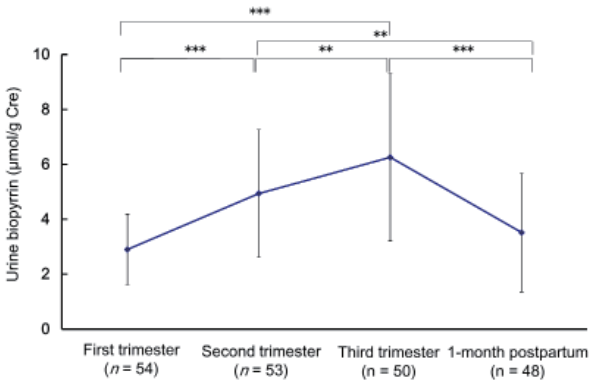

Figure 1. Urinary biopyrrin levels during pregnancy and at 1-month postpartum. Generalised linear mixed models involving the 4 assessment points as fixed effects and the subject as a random effect were established. Bonferroni's post-hoc comparisons were used to identify significant differences among the 4 assessment time points. Data are mean ± S.D. ** $p < 0.01$, *** $p < 0.001$

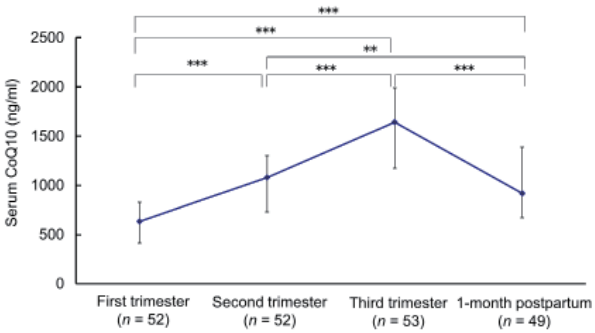

Figure 2. Serum Coenzyme Q 10 levels uring pregnancy and at 1-month postpartum. Generalised linear mixed models involving the 4 assessment points as fixed effects and the subject as a random effect were established. Bonferroni's post-hoc comparisons were used to identify significant differences among the 4 assessment time points. Data are mean ± S.D. ** $p < 0.01$, *** $p < 0.001$.

As shown in Table 2, the proportion of women who exercised (defined as more than 30 minutes, twice per week) was significantly lower during the first trimester (7.4%) than during the second and third trimesters (20% and 20%, respectively), but similar to that at 1-month postpartum (2.0%, $p = 0.007$). Interestingly, the proportion of women who worked during pregnancy changed significantly from 31.7% during the first trimester to 27.8% during the second trimester, 9.3% during the third trimester, and 2.0% during the first postpartum month ($p < 0.001$). Of these women, eight retired, six went on maternity leave, and two stopped working for another reason.

During the first trimester, 5 (9.3%) and 4 (7.4%) women took vitamin supplements (*e.g.*, vitamins C, E, B, and/or folic acid), on a regular and irregular basis, respectively. These proportions did not change significantly after delivery ($p = 0.15$). As shown in Table 3, daily food-related intake of vitamins, including folate and vitamins B6, B12, C, and E, did not change significantly from pregnancy to after delivery.

Table 2. Lifestyle Factors during pregnancy and at one month postpartum

Items	First trimester (n = 54)	Second trimester (n = 54)	Third trimester (n = 54)	1- month postpartum (n = 49)	p[1)]
Breakfast habits	43 (79.6)	40 (74.1)	44 (81.5)	40 (81.6)	0.87
Night-time sleep (h)	7.5 (4.5 - 10)	7.0 (5.0 - 9.0)	7.0 (3.5 - 10.0)	5.0 (3.0 - 7.0)	< 0.001***
Day time sleep (min)	30.0 (0 - 120)	37.5 (0 - 120)	30.0 (0 - 180)	30.0 (0 - 120)	0.41
Vitamin supplements intakes					
Regular intakes	5 (9.3)	8 (14.8)	4 (7.4)	0	0.15
Irregular intakes	4 (7.4)	2 (3.7)	3 (5.6)	3 (6.1)	
Alcohol consumption	2 (3.7)	2 (3.7)	3 (5.6)	2 (4.1)	0.96
Smoking habits	5 (9.3)	5 (9.3)	4 (7.4)	3 (6.1)	0.93
Number of cigarettes in a day[2)]	12.5 (5.0 - 20.0)	5.0 (2.0 - 15.0)	10.0 (3.0 - 20.0)	10.0 (8.0 - 10.0)	0.53
Exercise habits[3)]	4 (7.4)	11 (20.4)	11 (20.4)	1 (2.0)	0.007**
Number of exercise per week[4)]	5.3 (2.0 - 7.0)	4.0 (2.0 - 14.0)	5.0 (2.0 - 7.0)	3.0 [5)]	0.997
Current work	17 (31.7)	15 (27.8)	5 (9.3)	1 (2.0)	< 0.001***
Mental Health (GHQ score)[6)]					
0 - 12 points	1.0 (0 - 11)	0.0 (0 - 10)	0.0 (0 - 11)	1.0 (0 - 9)	0.11
≥ 4 points	13 (24.3)	4 (7.4)	8 (14.8)	10 (20.4)	0.14

Data are median (range; minimum - maximum) or n (%). [1)] **: $p < 0.01$, ***: $p < 0.001$. Numerical data: Generalized linear mixed models which contains 4 research points as fixed effect and subject as random effect was conducted. Categorical: χ^2 test or Exact test was conducted. [2)] Number of cigarettes smoked per day. [3)] At least two 30-min session per week. [4)] Number of exercises performed by those who routinely exercise. [5)] Real number in a pregnant woman who have exercise habits. [6)] GHQ: General Health Questionnaire. A high score indicates low mental health. n = 53 in second and n = 48 in 1-month postpartum for missing data.

Table 3. Daily dietary intake from first trimester to 1-month postpartum from BDHQ[1)]

Items	First trimester	Second trimester	Third trimester	1-month postpartum	p[4)]
n	54	54	53[2)]	44[3)]	
Folate (μg/1000kcal)	144.4 (60.7 - 294.7)	139.7 (74.9 - 339.7)	132.9 (80.6 - 311.2)	247.8 (72.8 - 247.8)	0.85
Vitamin B6 (mg/1000kcal)	0.6 (0.2 - 0.8)	0.6 (0.4 - 0.9)	0.6 (0.3 - 0.9)	0.6 (0.2 - 0.8)	0.68
Vitamin B12 (μg/1000kcal)	3.3 (0.2 - 7.5)	3.8 (1.2 - 15.2)	3.6 (0.7 - 9.2)	3.2 (0.6 - 5.6)	0.06
Vitamin C (mg/1000kcal)	51.4 (17.3 - 151.6)	48.3 (18.6 - 111.4)	46.3 (20.0 - 125.9)	51.6 (10.3 - 124.5)	0.71
Vitamin E (mg/1000kcal)	4.6 (2.2 - 7.2)	4.6 (2.4 - 7.7)	4.6 (2.6 - 7.1)	4.2 (2.3 - 6.0)	0.05
Amont of Alcohol consumption (g/1000kcal/day)	0 (0 - 7.0)	0 (0 - 1.1)	0 (0 - 1.2)	0 (0 - 9.7)	0.3
Polyunsaturated fatty acid intake (g/1000kcal/)	6.9 (3.9 - 10.9)	7.3 (4.1 - 12.3)	7.2 (4.4 - 11.1)	7.1 (3.7 - 9.2)	0.3

Data are shown at the Median (Range; Minimum - Maximum) or n (%). [1)] BDHQ; brief, self-administered, diet history questionnaire. [2)] Missing data for 1 out of 54 participants. [3)] Missing data for 5 of 49 participants. [4)] The generalized linear mixed model (which included the four time points as fixed effect and subject as random effect) was used.

Table 4. Lifestyle factors relating to levels of Biopyrrin by trimester of pregnancy

Items	First trimester (n = 54)		Second trimester (n = 52)[1)]		Third trimester (n = 49)[2)]		1- month postpartum (n = 42)[3)]	
	β	p	β	p	β	p	β	p
Breakfast habits (0: no, 1: yes)	- 0.33	0.04*	0.11	0.49	- 0.12	0.39	0.02	0.89
Smoking habits (0: no, 1: yes)	- 0.19	0.23	- 0.03	0.87	0.29	0.03*	0.66	< 0.001***
Amont of Alcohol consumption (g/1000kcal/day)	- 0.04	0.79	- 0.02	0.87	0.45	< 0.001***	- 0.02	0.89
Polyunsaturated fatty acid intake (g/1000kcal/day)	- 0.11	0.44	- 0.06	0.68	- 0.44	0.002**	0.12	0.41
Number of exercise per week (times/week)	0.22	0.14	- 0.24	0.11	0.09	0.49	- 0.09	0.53
GHQ Score: 0 - 12 points[4)]	0.29	0.045*	- 0.08	0.57	0.29	0.02*	- 0.06	0.65
Adjusted R^2	0.08	0.12	- 0.06	0.78	0.34	0.001**	0.31	0.003**

β: standardized partial regression coefficient. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ by multiple regression analysis. [1)] n = 52 in the second trimester with missing data for biopyrrin level and the GHQ. [2)] n = 49 in the third trimester with missing data for biopyrrin level (n = 4) and dietary status. [3)] n = 42 in the second trimester with missing data for biopyrrin level (n = 1), the GHQ (n = 1), and dietary status (n = 5). [4)] GHQ (General Health Questionnaire): the higher the score indicate the lower the mental health.

Tables 4 and 5 indicate the relationship between various lifestyle factors during each trimester and urinary biopyrrin levels at same points. Higher biopyrrin levels were significantly associated with non-consumption of morning meals during the first trimester ($p < 0.05$), smoking habits during the third trimester (p

Table 5. Lifestyle factors relating to levels of Coenzyme Q10 by trimester of pregnancy

Items	First trimester ($n = 52$)[1]		Second trimester ($n = 51$)[2]		Third trimester ($n = 52$)[3]		1- month postpartum ($n = 43$)[4]	
	β	p	β	p	β	p	β	p
Breakfast habits (0: no, 1: yes)	- 0.28	0.11	- 0.28	0.060	- 0.29	0.051	- 0.21	0.16
Smoking habits (0: no, 1: yes)	- 0.37	0.031*	- 0.37	0.014*	- 0.38	0.013*	- 0.55	0.001**
Amont of Alcohol consumption (g/1000kcal/day))	0.02	0.87	- 0.07	0.59	0.08	0.54	- 0.003	0.98
Polyunsaturated fatty acid intake (g/1000kcal/day)	- 0.08	0.61	0.13	0.34	- 0.18	0.20	- 0.28	0.06
Number of exercise per week (times/week)	0.24	0.11	0.24	0.09	0.36	0.012*	0.43	0.004**
GHQ Score: 0 - 12 points[5]	- 0.03	0.83	0.06	0.67	0.04	0.76	- 0.04	0.8
Adjusted R^2	0.05	0.22	0.11	0.07	0.16	0.03*	0.29	0.005**

β: standardized partial regression coefficient. *: $p < 0.05$, **: $p < 0.01$ by multiple regression analysis. [1] $n = 52$ in the first trimester with missing data for CoQ10 level. [2] $n = 51$ in the second trimester with missing data for CoQ10 ($n = 2$) and the GHQ ($n = 1$). [3] $n = 52$ in the third trimester with missing data for CoQ10 ($n = 1$) and dietary status ($n = 1$). [4] $n = 43$ in third trimester for missing data of GHQ ($n = 1$) and dietary data ($n = 5$). [5] GHQ (General Health Questionnaire): the higher the score indicate the lower the mental health.

< 0.05) and 1-month postpartum ($p < 0.001$), alcohol consumption during the third trimester ($p < 0.001$), high food-based polyunsaturated fatty acid intake during the third trimester ($p < 0.01$), and higher GHQ-12 scores during the first and third trimesters ($p < 0.05$, both trimesters). Higher CoQ10 levels were significantly associated with no smoking habits during all trimesters ($p < 0.05$) and 1-month postpartum ($p < 0.001$) and higher frequencies of exercise during the third trimester ($p < 0.05$) and 1-month postpartum ($p < 0.01$).

4. Discussion

The present study investigated changes in urinary biopyrrin levels during pregnancy and the immediate postpartum period in healthy Japanese women. The relationships between various lifestyle factors and markers of oxidative stress and antioxidant levels during each trimester and 1-month postpartum were analysed. The results showed significant increases in the levels of urinary biopyrrins (a marker of oxidative stress) and CoQ10 (an antioxidant) during pregnancy, with peak levels achieved during the third trimester. These results, in normal pregnant women, indicate the usefulness of urinary biopyrrins as markers of oxidative stress during pregnancy. These findings also add support to previously published results that demonstrated increased levels of oxidative products in healthy pregnant women, including lipid hydroperoxides (*29*), oxidised LDL (*30*), and malondialdehyde (*9*) in longitudinal studies as well as increased levels of urinary biopyrrins in cross-sectional studies (*31*). Furthermore, the findings of significant increases in antioxidant levels during pregnancy matched those reported for levels of vitamin E (*30*), TAC, uric acid (*29*), and CoQ10 (*32,33*).

Urinary biopyrrins and CoQ10 levels decreased significantly after delivery, relative to those recorded during the second and third trimesters. Previous studies reported that pregnancy is associated with a state of oxidative stress due to increased placental mitochondrial activity and production of ROS, primarily the superoxide anion (*34*). The placenta also produces other ROS such as nitric oxide and peroxynitrite, which have pronounced effects on placental function, including trophoblast proliferation and differentiation and vascular reactivity (*34*). We also previously reported that the mean level of urinary biopyrrins was 1.7 ± 0.9 μmol/g Cre in non-pregnant women (*31*), which is markedly lower than that found in pregnant women in the present study. Thus, the above studies and the present findings suggest that the reason for the decrease in the levels of both markers after delivery is related to the termination of pregnancy and the tissue that produced the ROS (i.e., the placenta).

The standardised partial regression coefficient (β) for the relationship between urinary biopyrrins and serum CoQ10 from the first trimester to 1-month postpartum was –0.303 ($p < 0.001$). In other words, there was a significant negative correlation between urinary biopyrrins and serum CoQ10 levels. We have also reported that the mean level of serum CoQ10 in non-pregnant women was 623 ± 237 ng/mL (*35*), which is markedly lower than that found in pregnant women in the present study. The elevated levels of antioxidants (*i.e.*, serum CoQ10) during pregnancy seem to counterbalance the heightened state of oxidative stress that also exists during pregnancy.

Urinary biopyrrin concentration was negatively correlated with the serum CoQ10 level. On the other hand, both urinary biopyrrin and serum CoQ10 levels increased until the third trimester and decreased at 1-month postpartum. Regardless of the negative correlation between the AUCs of both markers, the standard deviation of biopyrrin was greater than that of CoQ10. This indicates that participants with higher CoQ10 AUCs had lower biopyrrin AUCs throughout pregnancy and at 1-month postpartum. Our results showed that the levels of oxidative stress markers and

antioxidants were the highest at the third trimester. In this regard, Shimomura *et al*. (*36*) examined men and non-pregnant women during normal psychological changes and concluded that the maximum attainable increase in urinary biopyrrin levels was twofold that of the normal value. However, our results showed that the levels of urinary biopyrrins can increase by more than twofold during pregnancy. A comparison of the results from other studies also indicated that the third trimester was associated with increased oxidative stress, represented by the level of urinary biopyrrins during that period of pregnancy, than that reported following ultramarathons (4.05 μmol/g Cre) (*37*) and in patients with depression (4.7 μmol/g Cre) (*11*).

The present results showed that the higher biopyrrin and lower CoQ10 levels during each trimester were significantly associated with smoking habits during the third trimester and during the first month postpartum for biopyrrin, and during each trimester and the first month postpartum for CoQ10. A high frequency of exercise during the third trimester and the first month postpartum was significantly associated with higher CoQ10 levels. Higher biopyrrin levels during each trimester were also significantly associated with non-consumption of the morning meal during the first trimester, alcohol consumption during the third trimester, high food-based polyunsaturated fatty acid intake during the third trimester, and higher GHQ-12 scores during the first and third trimesters.

These results are in agreement with those of previous studies, which showed significantly higher plasma concentrations of malondialdehyde, a marker of oxidative stress ($p < 0.001$), and significantly lower levels of ascorbic acid, an antioxidant ($p < 0.001$), in men with high alcohol consumption than in men with low alcohol consumption (*10*). Furthermore, smoking during pregnancy has been reported to be associated with high levels of LOOH, malondialdehyde, and free radicals, and with low levels of catalase, TAC, vitamin E, and β-carotene in cord or maternal blood (*9*). High consumption of vitamin C-rich fruits and vegetables was reported to be independently and significantly associated with reduced oxidised LDL levels and with increased TAC and glutathione peroxidase activity in healthy young adults (*14*). The combination of balanced food and exercise has also been stressed. For example, a previous review concluded that regular exercise results in upregulation of antioxidant defence mechanisms, which help minimise oxidative stress (*13*).

The current study reported that the GHQ-12 total score decreased between the first and third trimesters; however, the decrease was not significant ($p = 0.11$; from the first trimester to 1-month postpartum). This result was consistent with our cross-sectional study of 594 pregnant women between their first and third trimesters. Therefore, it is feasible to assess the neurologic manifestations by the GHQ-12 questionnaire. Anan *et al*. also reported that only somatic symptoms in 4 subscales of the GHQ-28 decreased significantly from early (12-16 weeks) to late (32-26 weeks) gestation among healthy, pregnant, Japanese women; their mental health, including 'anxiety and insomnia' and 'severe depression', did not decrease (*38*). However, somatic symptoms would be expected to decline throughout pregnancy as pregnant women experience dramatic changes to their bodies. Moreover, previous studies have also reported that biopyrrin levels increase under stress conditions, such as poor mental health, in non-pregnant women or men (*11,12*). Our results showed that biopyrrin levels increase in association with lower mental health scores during pregnancy.

Although previous studies have also examined the relationship between lifestyle and oxidative stress or antioxidant capacity, the present study demonstrated that lifestyle factors during each trimester correlated with oxidative stress and antioxidant levels. These findings may be useful for future interventional studies aimed at preventing pregnancy-related complications in women and their newborns.

The present study has some limitations. First, we cannot generalize the results to the larger Japanese population because the participants were recruited from a single clinic in a Tokyo suburb with a small sample size. Therefore, many independent variables could not be analyzed by multiple linear regression. Second, self-reporting bias might affect the values of lifestyle parameters such as smoking, alcohol consumption, and dietary intake. Third, we could not explain why, in the first and second trimesters, urinary biopyrrin level showed no significant association with smoking habits. This result might indicate the presence of factors that were not analyzed, such as morning sickness or rapid placental and fetal growth in the first and second trimesters. Fourth, we used the total CoQ10 level to represent the antioxidant status of pregnant women without measuring levels of ubiquinone-10 and ubiquinol-10 separately because approximately 95% of total CoQ10 exists in the human circulation as ubiquinol-10 (*39*). However, we may have achieved greater accuracy in our results if we had measured the levels of ubiquinone-10 and ubiquinol-10 separately.

5. Conclusion

The present study confirmed that pregnancy represents a state of oxidative stress and elevated antioxidant levels, based on the observed biopyrrin and counterbalancing CoQ10 levels. We also speculate that certain lifestyle choices can reduce oxidative stress and increase antioxidant levels, such as the avoidance of alcohol consumption, smoking cessation, the maintenance of stable mental health, and regular exercise during pregnancy and the early postpartum periods.

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Case Report

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Primary hepatic mucosa-associated lymphoid tissue lymphoma and hemangioma with chronic hepatitis B virus infection as an underlying condition

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Summary **Primary hepatic mucosa-associated lymphoid tissue (MALT) lymphoma has a low incidence and is a rare subtype of hepatic malignant lymphoma. Described here is a rare case of primary hepatic MALT lymphoma and hepatic hemangioma with chronic HBV infection as an underlying condition. Possible treatment modalities, which should be selected in accordance with tumor size, tumor location, tumor number, and underlying liver disease, are discussed in conjunction with a review of the literature. In addition, the potential use of hepatic resection, radio frequency ablation (RFA), or radiotherapy followed by chemotherapy *via* the R-CHOP regimen is also discussed.**

Keywords: Primary hepatic mucosa-associated lymphoid tissue lymphoma, hepatic hemangioma, chronic hepatitis B virus infection, treatment modality

1. Introduction

Primary hepatic malignant lymphoma has an extremely low incidence, accounting for 1% of all malignant lymphomas, although secondary involvement of the liver is common in lymphoma (*1,2*). Primary hepatic mucosa-associated lymphoid tissue (MALT) lymphoma has an even lower incidence and is a subtype of hepatic malignant lymphoma. There are several case reports regarding hepatic MALT lymphoma with or without hepatitis C virus (HCV) infection, but there were only 2 cases involving hepatitis B virus (HBV) infection and no mentions of concurrent hepatic hemangioma or relevant treatment modalities. Described here is a rare case of primary hepatic MALT lymphoma and hepatic hemangioma with chronic HBV infection as an underlying condition. After surgery, this case was followed for 40 months. Here, possible treatment modalities are discussed.

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2. Case presentation

A 53-year-old Chinese male underwent a routine abdominal ultrasound (US) in January 2011. The patient had a history of HBV infection noted 20 years ago. He had been undergoing a routine physical examination including liver enzymes, concentrations of serum a-fetoprotein (AFP), and abdominal US every 6-12 months.

Routine abdominal US revealed a mass with a diameter of 4.5 cm in liver segments 4-8. This mass was identified as a malignant hepatic tumor based on contrast-enhanced US but could not be subgrouped into hepatocellular carcinoma (HCC) or other malignancies (Figure 1A). Another mass with a diameter of 1 cm was found on the surface of liver segment 5. This second mass was identified as hepatic hemangioma. Subsequent magnetic resonance imaging (MRI) was unable to subgroup this hepatic malignancy (Figure 1B). Serum AFP was 3.3 ng/mL, and liver function was Child-Pugh class A. There were no abnormal findings in gastroscopy and no gastric MALT lymphoma according to MRI.

The patient subsequently underwent a nonanatomic hepatic resection of the tumor in segments 4-8 and the tumor in segment 5 rather than a biopsy since HCC could not be ruled out and since the patient had an

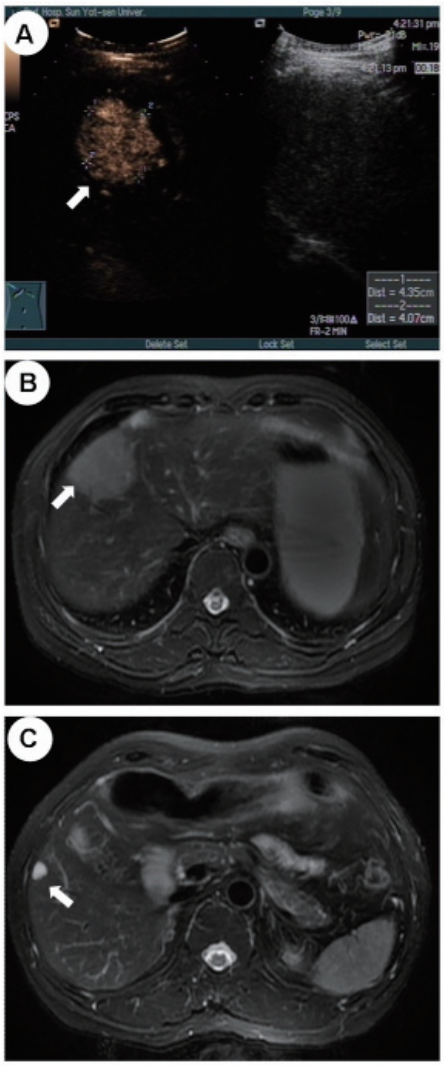

Figure 1. Preoperative imaging study. Contrast-enhanced ultrasonography (US) revealed a mass with a diameter of 4.5 cm, which was identified as a malignant hepatic tumor (white arrow), in liver segments 4-8 **(A)**; T2-weighted magnetic resonance imaging (MRI) revealed a mass in liver segments 4-8 **(B)** that could not be subgrouped and another mass in segment 5 that was diagnosed as hemangioma **(C)**.

acceptable liver reserve and no obvious liver cirrhosis. The surgical margin was > 1 cm. A specimen of the tumor from liver segments 4-8 is shown in Figure 2. Immunohistochemistry results indicated positivity for CD3 (+), positivity for CD45Ro (+), strong positivity for CD20 (+++), and strong positivity for CD79a (+++) (Figure 3). Based on its morphological and immunohistochemical features, this tumor was diagnosed as primary hepatic MALT lymphoma (low grade). The tumor in segment 5 was microscopically diagnosed as hepatic hemangioma according to hematoxylin and eosin staining.

The patient received 4 continuous courses of postoperative chemotherapy starting on March 1, 2011. The regimen was R-CHOP (rituximab 600 mg day 0, cyclophosphamide 1,000 mg day 1, pirarubicin 70 mg day 1, vincristine 2 mg day 1, prednisone 100 mg days 1-5). Once therapy ended the patient recovered

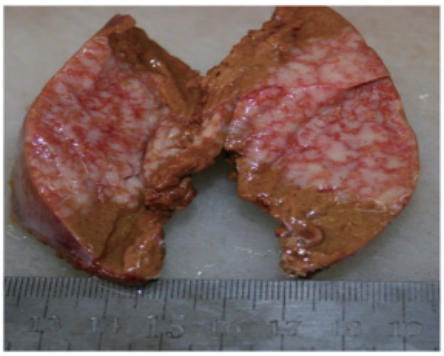

Figure 2. Specimen of the tumor from segments 4-8. Gross appearance of the tumor in liver segments 4-8. The cut surface of the tumor had a white-yellow medullary pattern.

uneventfully. No recurrence inside or outside of the liver was noted during follow up.

3. Review of the literature and discussion

The concept of MALT lymphoma in the gastrointestinal tract was first proposed by Isaacson and Wright in 1983 (*3*), and subsequent studies indicated that MALT lymphoma could involve many other sites, such as the thyroid glands, lungs, thymus, salivary glands, and liver, that are devoid of normal lymphoid tissue (*4-8*). MALT lymphomas account for approximately 7% to 8% of all non-Hodgkin lymphomas (*9*). Cases of hepatic MALT lymphoma with HCV infection have been reported, but cases without HCV infection have also been reported (*10-14*). Whether HCV infection can lead to hepatic MALT lymphoma or not remains unclear. There are only 2 reported cases of MALT lymphoma (*15,16*) with HBV infection. The correlation between HBV and hepatic MALT lymphoma remains unclear, although hepatic MALT lymphoma is thought to be associated with specific immune reactions or autoimmune disorders.

To date, the diagnosis of hepatic MALT lymphoma can only reached based on pathology and immunohistochemistry. There are no positive serum markers, and imaging studies such as MRI, computed tomography, and US do not yield patterns with features specific to hepatic MALT lymphoma (*17*).

The current patient was determined to have a malignant liver tumor preoperatively but this tumor could not be accurately diagnosed as hepatic MALT lymphoma, so the patient did not undergo a biopsy because of risk of possible needle seeding if the tumor was HCC. That said, low-grade lymphomas are characterized as indolent and having a favorable clinical course (*18*).

The clinical features of hepatic MALT lymphoma can affect the choice of treatment modality. Most cases of hepatic MALT lymphoma reported in the literature involve a hepatic resection. However, radiotherapy alone was performed in 1 case and resulted in long-

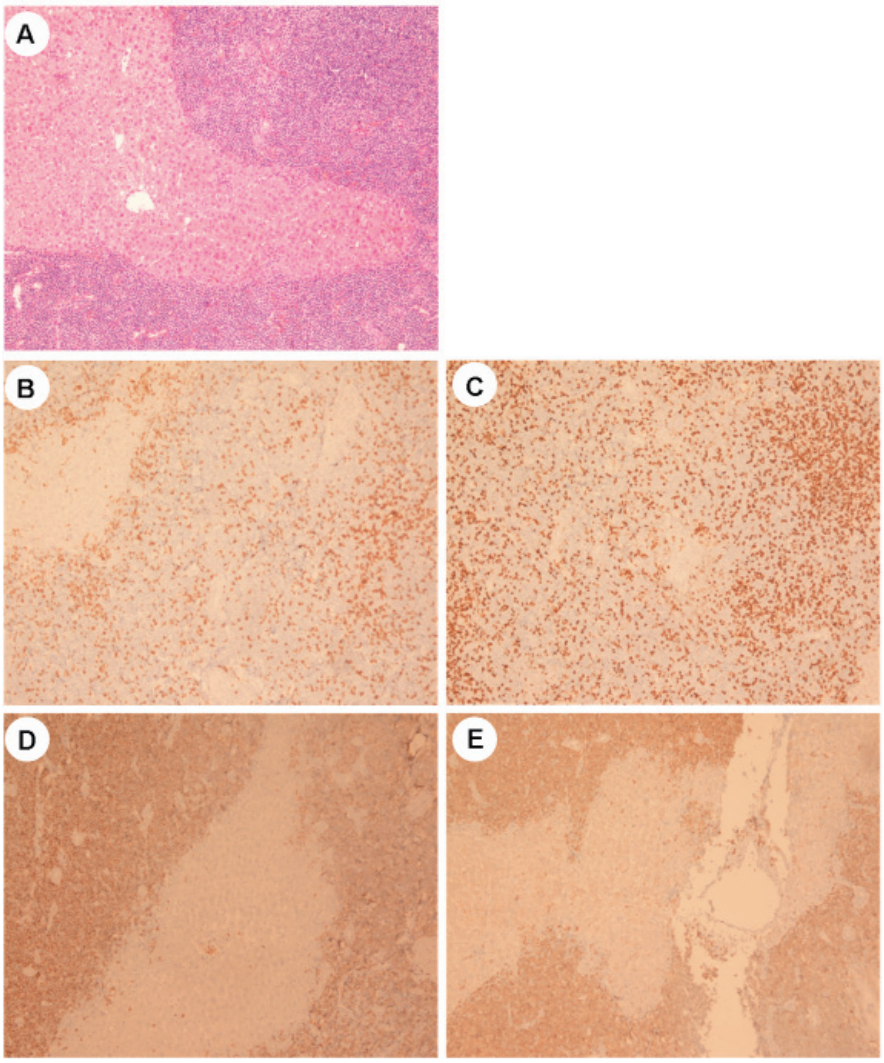

Figure 3. Microscopic examination of tumor specimens from liver segments 4-8 (×100). Lymphoid cells infiltrated along Gleason's sheath according to hematoxylin and eosin staining **(A)**. Immunohistochemistry indicated diffuse positivity for CD3 (+) **(B)**, diffuse positivity for CD45Ro (+) **(C)**, and strong positivity for CD20 (+++) **(D)** and CD79a (+++) (E).

lasting remission of 6 years (*19*). Radio frequency ablation (RFA) was performed in another case and subsequent rituximab chemotherapy resulted in a disease-free period of 24 months as of when the study was published (*20*). Since hepatic MALT lymphoma is a systemic form of non-Hodgkin lymphoma and based on reported cases, a hepatic resection was performed in the current case followed by 4 courses of chemotherapy *via* the R-CHOP regimen. Once therapy ended, the patient recovered uneventfully, and the patient has been disease-free for 40 months as of today. Rituximab is said to have a combination of immune-mediated effects, including complement-mediated lysis, antibody-dependent cell-mediated cytotoxicity, and direct effects induced by CD20 ligation leading to apoptosis (*21*). The CHOP regimen has been recommended for treatment of non-Hodgkin lymphoma (*22,23*). Radiotherapy, RFA, or hepatic resection might be selected as a local therapy to treat hepatic MALT lymphoma. As hepatic resection is the standard therapy for HCC and RFA is an option for small HCC, RFA and hepatic resection can reasonably be used to treat hepatic MALT lymphoma, which is more indolent than HCC. A needle biopsy should be performed on patients without HBV or HCV infection. A needle biopsy should also be considered for those with HBV or HCV infection, and it should be performed by an experienced doctor. Hepatic resection can be used to treat local hepatic MALT lymphoma without severe cirrhosis. RFA can be used to treat small hepatic MALT lymphoma (usually less than 3 cm in diameter) with severe cirrhosis. Radiotherapy can be attempted for diffuse or unresectable hepatic MALT lymphoma, and chemotherapy should subsequently be performed following the R-CHOP regimen since this regimen is effective at treating MALT lymphoma and hepatic MALT lymphoma cannot be ruled out as a systemic disease.

In conclusion, described here is a rare case of primary hepatic MALT lymphoma and hepatic hemangioma with chronic HBV infection as an underlying condition. A treatment modality should be selected in each case in accordance with the tumor size, tumor location, tumor

number, and underlying liver disease. Hepatic resection, RFA, or radiotherapy can be performed followed by chemotherapy *via* the R-CHOP regimen.

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Commentary

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Why are some HIV/AIDS patients reluctant to receive antiviral therapy as soon as possible in China?

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Summary **In more than 20 years of medical practice, a surprising phenomenon has often occurred: some patients with acquired immunodeficiency syndrome (AIDS) decide not to go to the hospital and they do not let others know that they are suffering from the disease unless they believe that they are dieing. Zhang Shan (a pseudonym) is one such patient with human immunodeficiency virus (HIV)/AIDS who was reluctant to receive antiviral therapy as soon as possible, and this paper shares Zhang's story as he related it. Clearly, there are numerous views as to why patients in China behave as Zhang did. Presented here are several reasons, including society, history, morality and ideology, family, and education. Although all of these reasons do play a role, the patient's mindset and behavior is the most significant reason for a patient's reluctance to seek treatment or disclose his/her status. If the individual patient's mindset and behavior are not dealt with effectively, then HIV/AIDS can continue to spread and threaten additional lives and even the fabric of society. This paper analyzes the reasons why patients are hesitant to receive antiviral therapy, but this paper also suggests steps healthcare personnel can take to encourage patients to seek treatment. Such steps can save the lives of current patients with HIV/AIDS. In addition, sound public health measures and a rational approach to treatment are important to helping potential patients with HIV/AIDS.**

Keywords: HIV/AIDS, antiviral therapy, China

Jonathan Baker, a Physician's Assistant in the human immunodeficiency virus (HIV)/ acquired immunodeficiency syndrome (AIDS) program in the division of infectious diseases at the University of Pittsburgh Medical Center, Pennsylvania recently published an interesting article entitled "Stay current with options for HIV prevention" (*1*). In the article, Baker stated that "conventional behavioral modification strategies have had limited effect on preventing the spread of HIV, and additional options are urgently needed. Antiretroviral drugs have been approved as preexposure prophylaxis, but vaccines and topical microbicides may provide additional options." Baker's article went on to review current HIV prevention options with a focus on biomedical prevention methods. Baker's sentiment is surely shared by other healthcare professionals who have long been fighting HIV/AIDS. However, the arduous job of prevention presents different challenges in different parts in the world.

The current article originated from a letter from a former patient with severe HIV infection who had only recently gotten his life back on track after he began to deal with his illness. His experience prompted the question of why some HIV/AIDS patients are reluctant to receive antiviral therapy as soon as possible in China.

The patient's name is Zhang Shan (a pseudonym). Zhang had returned to China from Southeast Asia in April 2010. Soon after returning, he began to feel unwell, he tired easily, he lacked energy, and he readily caught colds. Like most patients after a tortuous course, Zhang noticed trouble with his lungs and he finally went to see his doctor. On June 12, 2010, Zhang clearly and definitively tested positive for HIV. Zhang felt remorse, regret, helplessness, and despair at the time. When he was initially diagnosed, Zhang had a T lymphocyte count (CD4 T cells) of 49/μL and pulmonary tuberculosis as

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well. Specialists who considered Zhang's case felt that his prognosis was poor. After Zhang was definitely diagnosed, a treatment strategy was formulated to treat his tuberculosis first and then immediately start Zhang on antiretroviral therapy.

However, Zhang refused his doctor's advice based on his own thinking and he completely ignored doctor's instructions. Zhang did not believe that he could succumb to a virus that can only be seen under a microscope. As he reasoned, "If [the virus] is so easy for me to catch, then won't it be just as easy for my body to get rid of [the virus]? There must be a way, it just hasn't been found yet." Zhang similarly began to believe that he was capable of finding the real source (reason) of infection since doctors and scientists around the world had been unable to accurately describe the real source of HIV/AIDS. In Zhang's mind, all treatment was based on "assumptions" and thus less reliable. "Scientists and doctors are liars. I would never trust them," he said. Moreover, Zhang thought his situation was unlike the situations of others with the disease; he reasoned that his doctor may not have been able to cure him but may be could find a cure himself. Zhang wanted to find his own cure to the disease. Furthermore, Zhang was not content to take medication to control HIV/AIDS for the rest of his life. Instead, he would rather believe that doctors and scientists around the world were deceiving him rather than believing in fact. Zhang's preoccupation with curing HIV/AIDS himself brought him into conflict with personnel trying to treat him.

Zhang began to look for other possible cures for HIV/AIDS. After studying "all kinds" of AIDS-related knowledge, Zhang reached a "great conclusion" that "Western medicine is superficial and not to be trusted." "Since Western medicine can't offer me a cure, then I would rather give up on Western treatment," Zhang explained. Zhang then began to look closer at traditional Chinese medicine and he intensively studied medical information about cures for HIV/AIDS and related reports. He traveled across the country for almost a year in search of folk remedies. During that time, he met several famous "monks and Taoist priests" who claimed that they could cure him of his disease. Their treatments failed to work after almost a year, leaving Zhang penniless. Despair forced him return to this health center. Zhang's condition was grave. HIV/AIDS had taken a severe toll due to sepsis and drug allergies had almost been fatal. Zhang was infected with *Penicillium marneffei* and *Cryptococcus* and he had a T lymphocyte count (CD4 T cells) of only 8/μL. In the face of this cruel reality, Zhang lost all faith in his self-treatment. Zhang began to reconsider his original beliefs, and he realized the ridiculous and untenable nature of his previous thinking. Zhang finally discarded his old beliefs. Before dieing, Zhang wanted to try antiviral therapy. After three months of therapy, Zhang's condition gradually improved and he successfully cheated death. With his condition under control, Zhang's CD4 count rose to 176/μL. Half a year later, the virus was no longer detected and his CD4 count rose to 256/μL. A year later, his CD4 count rose to 363/μL. Zhang had survived.

This true story resulted in a highly gratifying result. Using scientific and standardized antiretroviral therapy to treat HIV/AIDS patients as soon as possible is crucial. The earlier treatment begins, the better! Antiretroviral therapy might not have been the treatment strategy Zhang wanted, but the fact remains that Zhang suffered because of his own behavior. There is no denying that some older regimens of active antiretroviral therapy (AAT) are still being administered in China and other developing countries (*2*). Moreover, the adverse effects of AAT usually result in opportunistic infections and complications (*3*). According to the 2012 China AIDS Response Progress Report by UNAIDS, China has a large population of people living with HIV/AIDS (PLHIV). Although AAT is the primary treatment for many potential sources of infection, traditional Chinese medicine has also been widely used as a complement to mitigate the adverse effects of AAT in China and other countries (*4,5*). Due to social beliefs and technical limitations, there is still no quantifiable standard governing the availability and toxicity of AAT. The low price of traditional Chinese medicines is the sole reason why they are used by a significant number of PLHIV in China (*6*). More clinical studies should be done to analyze traditional Chinese medicines in-depth. Drug administrators and policymakers should help PLHIV.

Medicine has made great strides in the development of HIV/AIDS antiretroviral therapies. Recent studies had highlighted the importance of eradicating HIV and curing AIDS. New views on antiretroviral therapies and multidisciplinary approaches to eradicating and curing HIV/AIDS have been described (*7*). One approach involves memory CD4 T-cells and seeks to utilize their stem cell-like properties to eliminate the viral reservoir in the hopes of achieving an AIDS-free world. Mounting research into stem cells has indicated that memory CD4 T-cells have stem cell-like properties and it has revealed their decisive role in antiretroviral therapies in the battle of modern medicine against HIV infection (*8*). This is in spite of the fact that both innate and adaptive immunities are indispensable and that numerous cells participate in anti-HIV immunity and antiretroviral therapy. Memory CD4 T-cells are nonetheless key cells that can organize all immune actions against HIV during antiretroviral therapy (*8*). Chinese clinicians and public health professionals were aware of and versed in advances made over the past few decades, but they view the current state of antiretroviral therapy and future challenges from a different perspective. The daunting task is to transform or change traditional or inculcated beliefs of potential AIDS patients so that they are open to antiretroviral therapy, which is a product of modern science, to help their plight.

The question is how to encourage patients to return to the hospital and receive antiretroviral therapy. Public health education will enhance the awareness of potential AIDS patients. Potential patients, however, face disparities in the current healthcare system. This is true in China as well as in other parts of Asia. Healthcare professionals are still in the learning phase in terms of guiding potential patients as they try to understand how to deal with affected individuals on a social level and as they try to bridge the gaps that cause disparities in access to care and effectiveness of treatment (*9,10*). Potential patients should be reminded of the words of Sir Isaac Newton: "If I have seen further, it is by standing on the shoulders of giants." Our clinicians, public health professionals, and educators/researchers have very important roles to play in eliminating disparities in behavior and mindset. Identifying challenges and barriers to healthcare in underserved communities is the first step in eliminating all disparities and providing tailored healthcare including antiretroviral therapy. Recently, the University of North Texas Health Science Center has actively worked to combat the problem of health disparities in Texas by promoting research, education, and training activities for underrepresented minorities (*i.e.* potential patients) (*11*). Clinicians, researchers, community workers, and other public health professionals noted how certain conditions disproportionally affect various minority populations. If such efforts were to be implemented in China, then there would be far fewer Zhangs.

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