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Policy Forum

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Quantum: May be a new-found messenger in biological systems

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Summary **Current studies on biological communications mainly focus on chemical signals. Since organisms are extremely complex, different kinds of signals may exist in the process of cell communication. The most probable candidate for alternative forms of organism communications is electromagnetic radiation, as many experiments have confirmed that electromagnetic radiation widely exists in cells, tissues, organisms and even between organisms and their surroundings. The well-known connection between electromagnetic radiation and quantization of the energy transfer makes us to suggest a bold, but fresh view that quantum can serve as a biological messenger. This view also coincides with the medium of Qi in the human body according to traditional Chinese medicine (TCM). Relating Qi with quantum may further explain a number of phenomena that cannot be explained solely by conventional chemical signaling systems.**

Keywords: Quantum, electromagnetic radiation, non-chemical, non-electrical, messenger, human body

Current studies on biological communications mainly focus on chemical signals like first, second, and third messenger signaling (*1*). As organisms are extremely complex, different kinds of signals may exist in the process of cell communication. Recently, electromagnetic radiation has been considered as the most probable candidate for alternative forms of organism communications (*2*), as many experiments have confirmed that electromagnetic radiation widely exists in cells, tissues, organisms and even between organisms and their surroundings. Electromagnetic radiation could contribute to a series of basic activities such as cell division and proliferation, oxidative metabolism, photosynthesis, and carcinogenesis (*2*). What follows is a brief review of studies on electromagnetic radiation effects in biological systems.

Scientific experiments related to the discovery of electromagnetic radiation in biological systems can be traced back to the 1920s and the work of Alexander G. Gurwitsch (*3*). Gurwitsch monitored the number of mitoses in a set of chemically isolated onion root cells that were near a group of actively dividing cells. He found that the number of mitoses increased if the chemically isolated roots were separated from actively dividing roots by quartz glass but not by normal glass. This suggested the existence of a form of cellular radiation in biological systems that Gurwitsch named "mitogenetic radiation." This study was the first to suggest that the emanation of light might play an important role in biological communication. Gurwitsch's observations stimulated early research on the ability of electromagnetic radiation to induce cell division. Moreover, a typical experimental model was set up to observe electromagnetic interactions in organisms (Figure 1) (*2*). Since then, many studies have observed quantum communication in biological systems.

Inspired by Gurwitsch's work, many researchers conducted experiments to confirm that electromagnetic radiation widely exists in cells of organisms. Kaznacheev *et al*. (*4*) found that the cytopathic effects of a cytopathic stimulus (*e.g.* UV radiation) in a cell culture plate could be observed in a distinct cell culture

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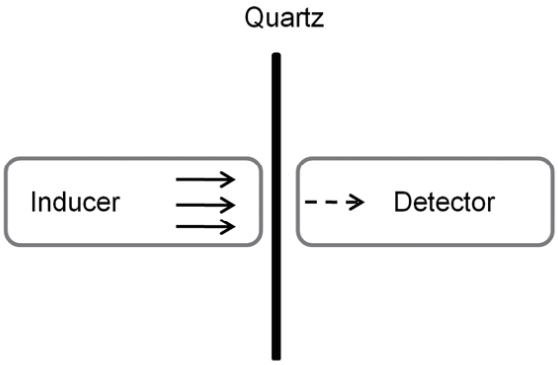

Figure 1. Typical model used in experiments to measure electromagnetic radiation communication in an organism. Inducer: a source of electromagnetic radiation; an organism emits electromagnetic radiation or is stimulated to emit electromagnetic radiation). Detector: detects how an organism reacts to electromagnetic radiation from the inducer. Quartz: a separator that ensures a chemical separation of the organism and detector to determine which part of the electromagnetic radiation spectrum is mediating the organism's interaction with the inducer and detector.

plate that was separated by quartz glass and not exposed to the cytopathic stimulus. Kaznacheev *et al*. called this phenomenon a "mirror cytopathic effect". They also found that cytopathic effects in the detector cells were only observed in those cells that were separated from the stimulus by quartz glass and not by regular glass. Their findings suggested that the mirror cytopathic effect was most likely due to ultraviolet or infra-red radiation emanating from the inducer. Grasso *et al*. (*5*) and Musumeci *et al*. (*6,7*) also observed a similar phenomenon in yeast cells. Furthermore, Albrecht-Buehler pioneered a series of experiments in which he used various cell culture models to investigate the effect of artificial and cellular infra-red electromagnetic radiation on cell functions and properties (*8*). He observed the interaction of baby hamster kidney cells separated from an inducer by a thin glass film. The cells (detector) on one side were able to determine the spatial orientation of the cells (inducer) on the other side through electromagnetic signals and the cells responded by adjusting their own orientation. He also found that 3T3 and CV1 cells were able to extend their pseudopodia towards microscopic infrared light sources nearby. Taken together, these findings suggest that the cells were able to sense specific infrared wavelengths and to determine the direction of light from individual sources.

Several experiments have also shown that electromagnetic radiation could contribute to cell proliferation and carcinogenesis in human cells. Zhang *et al*. (9) found that the quantum radiation emitted by rapidly proliferating osteoblasts promoted the proliferation of other osteoblasts. Farhadi *et al*. (*10*) found that exposing inducer cells (colon cancer cells CaCo-2) to H_2O_2 resulted in a significant reduction in total protein content, an increase in nuclear NFκB activation, and structural damage in detector cells that were placed in separate containers near the inducer cells but were not exposed to H_2O_2.

Influences of electromagnetic radiation in biological systems exist not only at the cell level but also at the level of tissues, organs, and organisms and even between organisms and their surroundings. In a study by Galantsev *et al*., two mammary explants of lactating mice were separated by quartz glass in a dish. One explant was treated with oxytocin, acetylcholine, epinephrine, or norepinephrine and the level of thiobarbituric acid-reactive substances (TBARS) that it produced changed; although separated by quartz glass, the untreated explant was also affected (*11*). Analysis of the level of TBARS formation in the two explants showed that their interactions might be associated with light emission resulting from lipid peroxidation processes. In a study by Yang *et al*., the biophotons from the palm and back of the hand of healthy people and stroke patients were observed and measured for a year (*12*). They found that the left-right balance of biophoton emission was maintained for normal subjects in contrast to the severe imbalance for stroke patients.

Electromagnetic radiation also plays an important role in other species, including bacteria and plants. Fels showed that *Paramecium caudatum* can interact in darkness *via* ultra-weak photon emission in both the UV and visible regions of the electromagnetic spectrum. He found that cells affected cell division and energy uptake in neighboring cell populations (*13*). Galle *et al*. suggested that using ultra-weak photon emission was an important way for insects to communicate. He found that adolescent *Daphnia magna* exhibited ultra-weak photon emission and that the intensity of electromagnetic radiation had a non-linear dependence on the population density with distinct maxima and minima (*14*). Kuzin *et al*. suggested that plant seeds also appeared to use ultra-weak photon emission to communicate (*15*). They stimulated *Raphanus sativus* seeds with low-dose gamma ray irradiation and found that the resulting germination and growth were also observed in detector seeds that were separated from the inducer seeds by quartz glass and not exposed to gamma rays. This effect was eliminated when the experiment was repeated using regular glass, which blocks the passage of ultraviolet photon emission.

In physics, a quantum is the minimum amount of any physical entity involved in an interaction (*16*). It reflects the fundamental notion of quantization of physical properties. Max Plank, who had been trying to understand the emission of radiation from heated objects, first discovered the concept of quantization of energy transfer in electromagnetic radiation in 1900 (*17*). This well-known connection between electromagnetic radiation and the concept of quantum, together with the various effects of electromagnetic

Table 1. Effects of electromagnetic radiation in biological systems

Researchers	Organism	Methods	Findings from detector	References
Gurwitsch	Onion root cells	Quartz or normal glass separation	The number of mitoses increased	(*3*)
Kaznacheev *et al*.	Fibroblasts (human & chicken), monkey kidney tissue	Various separator materials tested (*e.g.* quartz and glass)	Transfer of effect of high dose UV irradiation	(*4*)
Grasso *et al.*	Yeast cells	Air separation	The growth rate increased	(*5*)
Musumeci *et al*.	Yeast cells	Quartz glass separator	The growth rate increased	(*6,7*)
Albrecht-Buehler	BHK, CV1 and 3T3 cells	Various separator materials tested	Orientation to detector	(*8*)
Zhang *et al*.	Osteoblasts	Copper net separator	Proliferation promotion	(*9*)
Farhadi *et al*.	Colon cancer CaCo-2 cells	Kept in a distant laboratory with separation by walls and doors	A significant reduction in total protein content, an increase in nuclear NFκB activation, and structural damage	(*10*)
Galantsev *et al*.	Mammary explants of lactating mice	Quartz glass separator	The level of TBARS changed	(*11*)
Yang *et al*.	Palm and back hand of healthy people and stroke patients	Biophoton radiation	The left-right balance of biophoton emission was maintained for normal subjects in contrast to the severe imbalance for stroke patients.	(*12*)
Fels	*Paramecium caudatum*	Quartz or normal glass separator	Cell division and energy uptake	(*13*)
Galle *et al*.	*Daphnia magna*	Organisms in water environment	The intensity of electromagnetic radiation had a non-linear dependence on the population density	(*14*)
Kuzin *et al*.	*Raphanus sativus* seeds	Quartz glass separator	Seed germination and development	(*15*)

Abbreviation: TBARS: thiobarbituric acid-reactive substances.

radiation in biological systems (which have been summarized in Table 1) makes us to suggest a bold, but fresh view that quantum may be a new-found messenger in biological systems.

Interestingly, the view that quantum can be a biological messenger coincides with the medium of Qi in the human body according to traditional Chinese Medicine (TCM). Because, on the one hand quanta are considered to be discrete packets with energy stored in them, on the other hand, according to TCM, Qi is one of the most fundamental substances in the human body and in its maintenance of biological activities. In particular, Qi connects the viscera (Zang-Fu) with meridians or channels (Jing-luo) of the body that promote and stimulate the physiological functions of the human body (*18*). Although still in a preliminary stage, the quantum explanation of biological communications has been used to explain much of the TCM theory related to Qi, including "adaptation of the human body to the natural environment" and "treatment based on syndrome differentiation" (*19*). Furthermore, by combining Qi and quantum theories, maybe we can establish a discipline called Qunatum TCM theory in the future, which may provide a new direction for research on biological systems including human bodies.

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Brief Report

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Affinity of anti-insulin-like growth factor I receptor antibody binding to the receptor altered by plant lectins

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Summary **The binding ability of anti-insulin-like growth factor I receptor (IGF-IR) single-chain variable fragments (scFvs) to IGF-IR was measured in the presence of plant lectins. Combinations of concanavalin A (Con A), wheat germ agglutinin (WGA), or peanut agglutinin (PNA) and 1H7 or 3B7 anti-IGF-IR scFv/phage antibodies that were previously produced and characterized were used. WGA inhibited binding of both scFvs proteins to the receptor. PNA slightly enhanced the binding of 1H7 scFv and phage antibody to the receptor. Con A led to enhancement of 3B7 scFv-binding but had no effect in a test of phage antibodies and determination of kinetic parameters. The effect of lectins differed for scFvs and phage antibodies, implying that affinity altered by lectins is dependent upon the molecular structure of the antibodies. Results indicated that animal lectins may affect the affinity of therapeutic antibodies targeting cell membrane receptors *in vivo*.**

Keywords: Altered affinity, scFv, IGF-IR, lectin

1. Introduction

Insulin-like growth factor I receptor (IGF-IR) is a hetero-tetrameric glycoprotein that consists of two α subunits and two β subunits. The α subunit is completely extracellular whereas the β subunit is a transmembrane protein with a cytoplasmic tyrosine kinase domain. When ligands like IGF-I and IGF-II bind to the receptor, tyrosine kinase is autophosphorylated and activated, leading to activation of down-stream signaling molecules including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K). This leads to biological action like the induction of proliferation or inhibition of apoptosis (*1,2*).

IGF-IR is overexpressed in various types of tumors and is thus considered to be a target molecule for cancer therapy (*3*). Several strategies targeting IGF-IR have been developed, including IGF-IR specific antibodies (*4,5*), tyrosine kinase inhibitors (*6*), small interference RNA (*7*), and a dominant negative type of IGF-IR (*8*). Of these, anti-IGF-IR antibodies are probably the best strategy for cancer therapy. The current authors previously reported that a chimeric scFv-Fc consisting of mouse anti-IGF-IR single-chain variable fragments (scFv) and a human IgG_1 Fc domain inhibits tumor growth *in vivo*; this tumor inhibition was attributed to IGF-IR down-regulation (*9,10*). More recently, the current authors produced scFvs derived from original monoclonal antibodies (mAb) that either inhibits growth (1H7) or has a stimulatory effect (3B7) (*11,12*), and determined the affinity and epitope specificity of 1H7 and 3B7 scFvs (*13*). Several other researchers also reported on the action of anti-IGF-IR antibodies with an eye towards the development of anti-IGF-IR antibody therapeutics (*14-16*).

A variety of proteins that recognize carbohydrate moieties play important roles in animals. Mannan-binding lectin (MBL), L-ficolin, M-ficolin, and H-ficolin are all complement-activating soluble pattern recognition molecules that play critical roles in human innate immunity (*17*). MBL and ficolins are reported to play a role in the clearance of apoptotic cells (*18,19*). Galetins are a family of proteins that bind to

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the β-galactoside carbohydrate structure through their carbohydrate recognition domains (*20*). These proteins have been shown to be involved in multiple biological functions such as cell-matrix and cell-cell interactions, cell proliferation, cell differentiation, cellular transformation, or apoptosis mainly through the way in which they bind to specific ligands. Partridge *et al.* reported that galectin-3 (soluble protein) can bind to the carbohydrate structures of EGF and TGF-β receptors that are modified by a specific Golgi enzyme (*21*). The multivalence of galectin-3 can create a lattice formation of those receptors, resulting in delays of receptor removal by constitutive endocytosis. Therefore, the lattice ensures up-regulation of surface receptors and increased sensitivity to growth factors (*21*). Those reports strongly suggest that circulating carbohydrate binding molecules may modify functions of cell surface receptors by binding to carbohydrate moieties on the receptors.

Plant lectins are known to alter the relationship between hormones and their receptors on cell membranes. For example, Buxser *et al.* reported that the binding of nerve growth factor to human melanoma cell membranes significantly increased when binding was carried out in the presence of wheat germ agglutinin (WGA) (*22*) and Masnikosa *et al.* reported that binding of IGF-II to its receptor increased in the presence of WGA (*23*).

Based on these studies, a hypothesis, *i.e.*, that animal lectins affect the dynamics of therapeutic antibodies targeting cell membrane receptors *in vivo*, was put forward. Therefore, model experiments were performed to test whether anti-IGF-IR scFv affinity for IGF-IR changes in the presence of plant lectins. This study clearly demonstrated that plant lectins significantly affect scFvs affinity *in vitro*. The results of this study thus suggest that the affinity of therapeutic antibodies targeting receptors may be modified by circulating animal lectins. This important finding needs to be addressed when developing therapeutic antibodies.

2. Materials and Methods

2.1. *Materials*

Recombinant human extracellular domain of IGF-IR (rhIGF-IR) and insulin receptor (rhIR) were purchased from R&D Systems Inc. (MN, USA). Concanavalin A (Con A), WGA, and peanut agglutinin (PNA) labeled with biotin were obtained from Seikagaku Biobusiness (Japan). Horse radish peroxidase (HRP) conjugated with streptavidin, HRP-conjugated anti-E tag, and anti-M13 antibody were obtained from GE Healthcare (Piscataway, NJ, USA). 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)/H_2O_2 was from Roche Diagnostics (Mannheim, Germany).

2.2. *Selection and preparation of lectins*

Three carbohydrate-binding plant lectins, Con A, WGA, and PNA, were used in this study. Con A is a homotetramer of 26.5 kDa-subunit with a binding site for α-D-mannosyl and α-D-glucosyl residues (*24*). WGA exists as a heterodimer with a molecular weight of approximately 38 kDa and selectively recognizes *N*-acetylglucosaminyl residues that are predominantly found on the plasma membrane (*25*). PNA is a homotetramer of approximately 27 kDa-subunit and is galactose-specific and had strong anti-T-antigen activity (*26*). Commercially available biotin-labeled lectins were dissolved in phosphate buffer saline pH 7.4 (PBS) according to the manufacturer's instructions to yield stock solutions with final concentrations of 1 mg/mL.

2.3. *Preparation of scFvs or phage antibodies*

Phage antibodies and scFvs derived were produced from mouse monoclonal antibodies as previously described (*13*). 1H7 and 3B7 were scFvs in a VL-VH orientation whereas 1H7R and 3B7R were scFvs in a VH-VL orientation. For preparation of phage antibodies, *E. coli* cells were cultured in 40 mL of SBSC at room temperature for 2 h, followed by infection with 8.8×10^{10} pfu M13KO7 helper phage for 1 h at 37ºC. Infected *E. coli* was selected by culturing in the presence of 50 μg/mL kanamycin at 25ºC for 2 d. The culture supernatants containing phages were precipitated in 4% polyethylene glycol/0.5 M NaCl (PEG precipitation) and resuspended in 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl (TBS) with 1.5% BSA and 0.2% Tween-20 followed by treatment with benzonaze (Novagen) to digest any unnecessary DNA. The titer (cfu) of each phage was determined by means of infection activity against *E. coli* TG1 cells. Each resulting phage was subjected to ELISA.

For preparation of scFvs, *E. coli* cells were cultured overnight in 5 mL of 2 × YT medium containing 50 μg/mL carbenicillin, 50 μg/mL spectinomycin (2 × YTCS), and 1% glucose at 25ºC. The overnight culture was inoculated in 40 mL of freshly-prepared 2 × YTCS and incubated for 1 h at 30ºC, followed by induction with 1 mM IPTG at 30ºC for 5 h. Periplasmic fractions that contained soluble scFvs were collected by the osmotic shock method as described (*13*).

2.4. *Binding assay of lectins to rhIGF-IR or rhIR determined by ELISA*

Each well of a 96-well plate was coated with 250 ng/50 μL of rhIGF-IR or rhIR followed by incubation for 2 h at room temperature. Antigen-coated wells and control wells were blocked by incubation overnight

with 150 µL of 3% BSA/TBS at 4ºC. The wells were then incubated with 50 µL of various lectins labeled with biotin at room temperature for 2 h. The wells were washed 3 times with 150 µL of TBS containing 0.2% Tween-20 (TBST). For detection of lectins, HRP-conjugated streptavidin (1:1,000 dilution) was added to the wells and incubated at room temperature for 1 h. The wells were washed 7 times with TBST and then 3 times with TBS. Peroxidase activity was detected by reaction with 100 µL of ABTS/H_2O_2 for 30 min and termination with 1% oxalic acid. The absorbance at 415 nm was measured by a plate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA).

2.5. *Affinity of scFvs or phage antibodies binding to rhIGF-IR altered by lectins*

Antigen-coated wells were prepared and blocked by BSA and incubated with Con A, WGA, or PNA as described above. ScFvs or phage antibodies were then added to the wells and incubated at room temperature for 2 h. The wells were washed 3 times with TBST. For detection of bound scFvs or phage antibodies, HRP-conjugated anti-E tag antibody or anti-M13 antibody, respectively, was added to the wells and incubated at room temperature for 1 h. Peroxidase activity was detected as described above.

2.6. *Determination of kinetic dissociation constants*

ELISA-based measurements of the dissociation constant (K_D) were performed as described previously (*26,27*). Briefly, scFvs were incubated with excess concentrations of antigens or antigens pre-incubated with Con A (1 µg/mL), WGA (100 µg/mL), or PNA (100 µg/mL) at 4ºC overnight. Equilibrated antibodies/antigen complexes were transferred to wells coated with antigens or antigens pre-incubated with WGA that were blocked with BSA. After incubation at room temperature for 1 h, the wells were washed and bound antibodies were detected by adding secondary antibodies as described above. The value of K_D was determined as previously described (*26,27*).

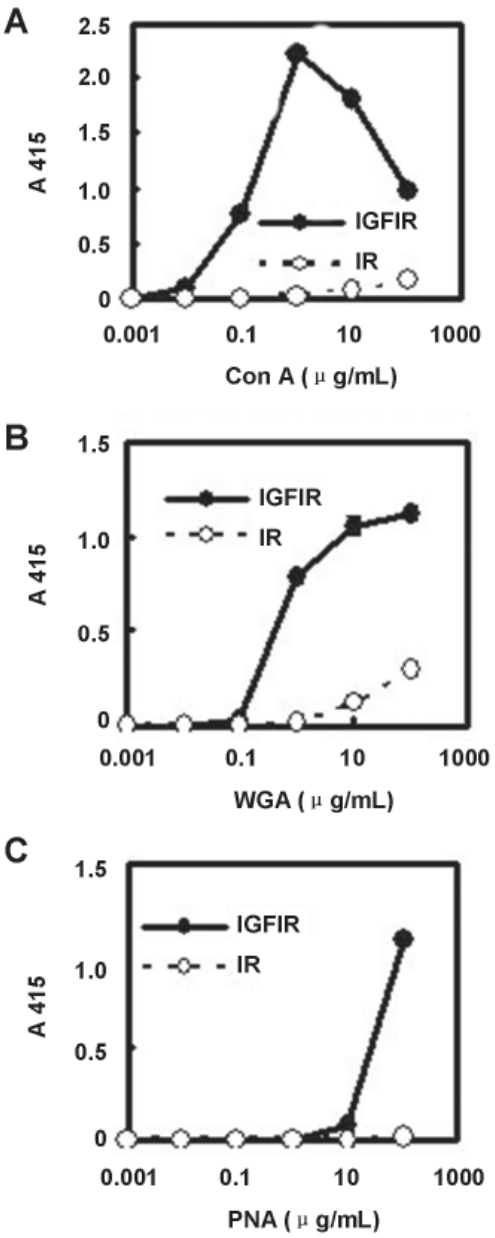

Figure 1. Binding assays of lectins to rhIGF-IR or rhIR as determined by ELISA. Binding of Con A **(A)**, WGA **(B)**, or PNA **(C)** to IGF-IR (●) or IR (○) was determined by ELISA as described in the "Materials and Methods". The error bars represent the standard deviation (n = 3).

3. Results and Discussion

First, the binding of three plant lectins to IGF-IR or IR was investigated. As shown in Figure 1, Con A (Figure 1A), WGA (Figure 1B), and PNA (Figure 1C) all bound to rhIGF-IR in a dose-dependent manner. Surprisingly, there was significantly little binding of three lectins to rhIR. This could be due to the recombinant IR used, which may differ from native IR in terms of glycosylation. Although the results of lectin binding to IGF-IR and IR were strikingly different, the positive results with IGF-IR were utilized to evaluate the effects of these lectins on scFv binding to rhIGF-IR.

Affinity of 1H7 and 3B7 scFvs in the presence of three concentrations of lectins was then examined. Con A did not significantly affect the affinity of scFvs except at 1 µg/mL (Figure 2A). WGA at 100 µg/mL significantly inhibited both 1H7 and 3B7R binding (Figure 2B). PNA at 10 and 100 µg/mL slightly enhanced 1H7 affinity but did not affect the binding of 3B7R scFv (Figure 2C).

To determine whether affinity altered by plant lectins depends on the molecular structure of antibodies, the same experiment as in Figure 2 was performed using phage antibodies expressing scFv proteins in two orientations – 1H7R or 3B7R in VH-VL

and 1H7 or 3B7 in VL-VH and lectin concentrations that affected the binding of 1H7 or 3B7R scFv protein to the receptor. With 1H7 and 1H7R phage antibodies, enhancement by PNA and inhibition by WGA were observed (Figures 3A and 3B) with 1H7 scFv (Figure 2). In contrast, slight enhancement by Con A and inhibition by WGA were observed with 3B7R phage antibody, but neither action was observed with 3B7 phage antibody (Figures 3C and 3D).

To further study the effects of lectins on the binding of scFv proteins to rhIGF-IR, affinity constants for binding of 1H7 and 3B7R scFv proteins to the receptor were determined in the presence or absence of the three lectins (Table 1). The control K_D values of 1H7 and 3B7R scFv in the absence of lectins were 16.4 ± 0.8 and 13.2 ± 2.0 nM, respectively. In the presence WGA, the K_D values of 1H7 and 3B7R scFv increased to 196 ± 40.0 and 41.0 ± 2.2 nM, respectively, indicating lower affinity. In the presence of PNA, the K_D value of 1H7 scFv slightly decreased to 9.31 ± 0.71 nM, indicating a slightly higher affinity. In the presence of Con A, however, the K_D value of 3B7R changed little from the control. These results coincide with those originally observed (Figure 2).

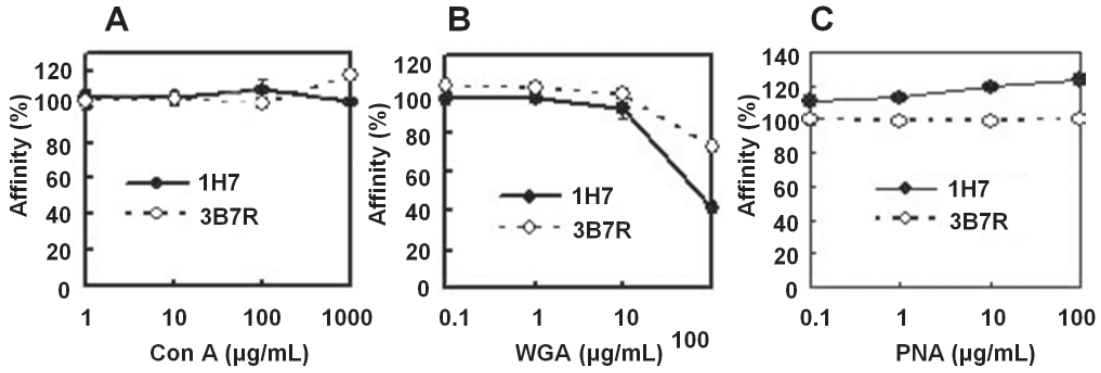

Figure 2. Alteration of anti-IGF-IR scFv protein binding to IGF-IR by lectins. Binding of 1H7 (●) or 3B7R (○) scFv proteins to rhIGF-IR in the presence of Con A **(A)**, WGA **(B)**, or PNA **(C)** at indicated concentrations was measured by ELISA as described in the "Materials and Methods". The error bars represent the standard deviation (n = 3).

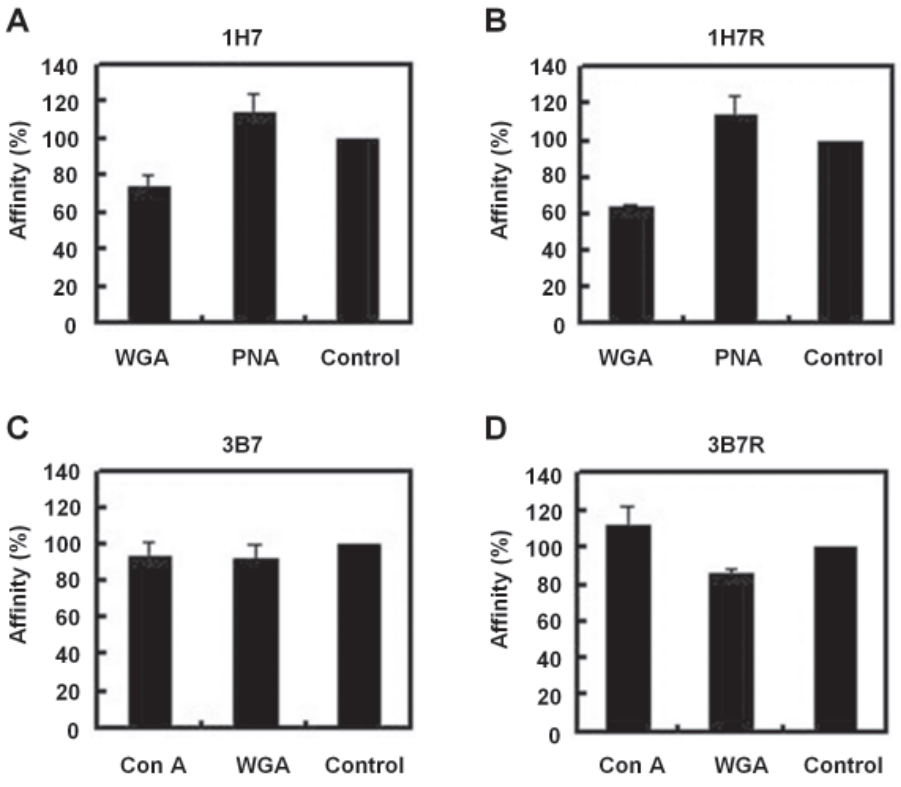

Figure 3. Alteration of anti-IGF-IR phage antibody binding to IGF-IR by lectins. Binding of 1H7 **(A)**, 1H7R **(B)**, 3B7 **(C)**, or 3B7R **(D)** phage antibodies to rhIGF-IR in the presence of Con A at 1 μg/mL, WGA at 100 μg/mL, or PNA at 100 μg/mL was measured by ELISA as described in the "Materials and Methods". The error bars represent the standard deviation (n = 3).

Table 1. Kinetic parameters of 1H7 and 3B7R scFvs in the presence or absence of lectins

Treatment	1H7 scFv	3B7R scFv
WGA (+)	196 ± 40.0	41.0 ± 2.2
PNA (+)	9.31 ± 0.71	ND
Con A (+)	ND	16.6 ± 1.7
Control	16.4 ± 0.8	13.2 ± 2.0

ND: Not determined. The dissociation constant (K_D, nM) was determined by ELISA as described in the "Materials and Methods".

This study demonstrated that plant lections that bind carbohydrate moieties on IGF-IR alter the affinity of anti-IGF-IR antibodies binding to the receptor. The most significant effect observed was that when the receptor was treated with WGA. WGA at 100 μg/mL significantly inhibited the binding of both 1H7 and 3B7 scFvs to the receptor. The inhibitory effect of WGA was also observed with 1H7, 1H7R, and 3B7R phage antibodies but not with 3B7 phage antibody to the receptor. A possible conformational difference due to the opposite orientation of VL and VH may have resulted in this difference. The presence of PNA slightly enhanced the binding of 1H7 scFv as well as 1H7 and 1H7R phage antibodies to the receptor. The changes in affinity observed in the presence of lectins could be due to changes in receptor conformation or steric hindrance. Con A slightly stimulated 3B7R scFv-binding to the receptor, which was confirmed by experiments with 3B7R phage antibodies and K_D measurements. Although this study used a model system, the results indicated that binding kinetics of therapeutic anti-IGF-IR antibodies to the receptor on cancer cells may be altered by animal lectins.

In conclusion, this *in vitro* study suggests that therapeutic antibodies targeting cell membrane receptors may be altered *in vivo* in terms of their affinity or action by circulating carbohydrate-binding proteins.

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Brief Report

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No association between *Vitamin D receptor* gene polymorphisms and nasopharyngeal carcinoma in a Chinese Han population

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Summary **An abundance of candidate genes have been reported as susceptibility factors for the risk of nasopharyngeal carcinoma (NPC). Vitamin D receptor (VDR) plays an important role in cellular differentiation and the control of proliferation in a variety of cell types. To our knowledge, however, no study has reported the relationship between the VDR and NPC. The purpose of this study is to explore the potential correlation between single-nucleotide polymorphisms of the VDR gene (*VDR*) *Fok* I and *Bsm* I and NPC. A total of 171 patients with NPC and 176 age- and sex-matched controls were involved in this study. Genotypes were determined by using polymerase chain reaction-restriction fragment length polymorphism and DNA sequencing. There were no significant differences in the genotype and allele frequencies of *VDR Fok* I and *Bsm* I polymorphisms between the group of patients with NPC and the control group in a Chinese Han population (for *VDR Fok* I: adjusted OR 1.03, 95% CI: 0.76-1.41; for *VDR Bsm* I: adjusted OR 0.80, 95% CI: 0.48-1.33). Further studies will be needed to explore the complicated gene-gene interaction and gene-environmental interactions in the susceptibility to NPC, especially in ethnically disparate populations in cohort study samples.**

Keywords: Vitamin D receptor, gene polymorphism, nasopharyngeal carcinoma

1. Introduction

Nasopharyngeal carcinoma (NPC) is a disease with a remarkable racial differentiation. It occurs relatively rare in the United States with an incidence rate generally less than 1/100,000, but it is autochthonic in Southern China and Southeast Asia with an aged-adjusted incidence rate shown to be 30-50/100,000 (*1*). NPC is a malignancy arising from the epithelial cells lining the nasopharynx and it is a leading cause of cancer deaths in the southern China and also is the 8th greatest cause of cancer mortality around China (*2*). Previous studies show that the incidence of NPC is associated with the combined effects of environmental carcinogens, such as tobacco smoking, consumption of salted fish, and biologic factors, including Epstein-Barr virus (EBV) infection and genetic susceptibility (*1,3,4*). However, the molecular mechanism of NPC pathogenesis is not yet well known. An abundance of recent research has shown single-nucleotide polymorphisms (SNP), such as cytochrome P450 2E1 (CYP2E1) (*5*), cyclin D1 (*6*) and a series of cytokines involved in the development of NPC (*7,8*), which indicated that NPC carcinogenesis may be due to genetic differences such as SNP.

For more than a century, Vitamin D has been recognized as playing a key role in the normal development and mineralization of a healthy skeleton. However, recent studies showed that more extensive roles for vitamin D were discovered in tissues through the vitamin D receptor (VDR), which is a member of the steroid hormone receptor superfamily. VDR has been found in most tissues and cells and it could arise in a wide variety of biologic responses to $1_{\alpha},25(OH)_2D_3$. The functional roles of VDR suggest that vitamin D plays additional roles in cellular differentiation and the control of proliferation in a variety of cell types (*9*).The VDR gene (*VDR*) is located on chromosome

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12q12-q14. Two common *VDR* SNPs were investigated intensively for association with various human traits, including *Fok* I (in exon 2) and *Bsm* I (in intron 8), (rs10735810, and rs1544410, respectively) (*10,11*). Recent studies suggested that polymorphisms of the VDR gene have been associated with several forms of cancer, such as breast cancer (*12*), prostate cancer (*13*), and colorectal cancer (*14*).

In our previous studies, we have found that gene polymorphisms of some cytokines may contribute to the development of NPC (*7,8*), such as *interleukin-1B*, *interleukin-8*, *interleukin-10*, and *transforming growth factor-b1*. Therefore, we hypothesized that VDR gene polymorphisms may also be involved in modulating susceptibility to NPC. Therefore, we conducted a hospital-based case-control study to explicate the potential association between *VDR Fok* I and *Bsm* I polymorphisms and the risk of NPC in a Chinese Han population.

2. Materials and Methods

2.1. *Study population*

171 NPC patients and 176 non-cancer controls were recruited in this study. All the patients and controls were unrelated Chinese people, and were selected from the same population living in China between July 2005 and March 2007 (Table 1). All the patients were recruited from West China hospital, Sichuan University. Pathologically confirmed NPC diagnosis was the only selection criterion for patients. The case group (131 males and 40 females) had a mean age (S.D.) of 46.4 (13.2) years. The control group consisted of 176 healthy volunteers who visited the general health check-up division at West China Hospital, Sichuan University. The mean age (S.D.) of the control group (126 males and 50 females) was 44.4 (13.4) years. Selection criteria for controls were no evidence of any personal or family history of cancer or other serious diseases. There was no significant difference between patients and control subjects in terms of gender and age distribution. Written informed consent was obtained from all subjects, and the study was performed with the approval of the ethics committee of Chinese Human Genome.

Table 1. Characteristics of the study population

Variables	NPC patients (n = 171)	Controls (n = 176)
Age(yrs)	46.4 ± 13.2	44.4 ± 13.4
Sex		
Male	131 (76.6)[a]	126 (71.6)
Female	40 (23.4)	50 (28.4)
Clinical stages		
stages I and II	21 (12.3)	–
stages III and IV	150 (87.7)	–
Histologic type (%)		
Poorly differentiated SCC	141 (82.5)	–
Undifferentiated cancer	22 (12.9)	–
Others	8 (4.6)	–

Abbreviation: SCC, squamous cell carcinoma. [a] Numbers in parentheses denote percentage; others include poorly differentiated adenocarcinoma (n = 2) and moderately differentiated SCC(n = 6).

2.2. *Genotyping*

Genomic DNA was extracted from circulating blood with an extraction kit (Bioteke Corporation, Beijing, China). The *VDR Fok* I (ref SNP ID: rs10735810) and *VDR Bsm* I (ref SNP ID: rs1544410) polymorphisms were detected with polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis, as described previously (*15,16*). Approximately 10% of the samples were sequenced to validate the results. In the absence of the restriction site, *Fok* I and *Bsm* I genotypes were defined by capital letters (F and B, respectively), and where the restriction site was present the two genotypes were defined by small letters (f and b, respectively).

2.3. *Statistical analysis*

The SPSS statistical software package (version 11.5) was used for all the statistical analyses. Genotype and allele frequencies of *VDR Fok* I and *Bsm* I were compared between NPC cases and controls using the chi square test and Fisher's exact test when appropriate, and odds ratio (OR) and 95% confidence intervals (CI) were calculated to assess the relative risk conferred by a particular allele and genotype. Demographic and clinical data between groups were compared using the chi square test and Student's t test. Hardy-Weinberg equilibrium was tested with a goodness of fit the for chi square test with one degree of freedom to compare the observed genotype frequencies among the subjects with the expected genotype frequencies. Statistical significance was assumed at the $p < 0.05$ level.

3. Results and Discussion

The genotype and allele frequencies of *VDR Fok* I and *Bsm* I polymorphisms between the controls and the cases are shown in Table 2. Genotype distributions of the two polymorphisms were found to be in accordance with the Hardy-Weinberg equilibrium expectation. The frequencies of the FF, Ff and ff genotypes of *VDR Fok* I were 31.3, 44.3, and 24.4% in controls, and 29.2, 46.8, and 24.0% in the cases. The frequencies of F and f alleles of *VDR Fok* I were 53.4 and 46.6% in controls and 52.6 and 47.4% in the cases. The frequencies of the bb and BB carrier genotypes of *VDR Bsm* I were 81.2 and 18.8 % in controls, and 84.2 and 15.8% in the cases. The frequencies of b and B alleles of *VDR Bsm* I were 89.8 and 10.2% in controls, and 91.8 and 8.2% in the cases. No significant differences were observed in the genotype distributions and allele frequencies of the

Table 2. The genotype and allele frequencies of *Fok* I and *Bsm* I in the VDR gene between patients with NPC and controls

Polymorphisms	Patients $n = 171$ (%)	Controls $n = 176$ (%)	Adjusted[b] OR (95% CI)	p
Fok I				
FF	50 (29.2)	55 (31.3)	1.00 (Ref)	0.63
Ff	80 (46.8)	78 (44.3)	1.11 (0.68-1.83)	0.87
ff	41 (24.0)	43 (24.4)	1.00 (0.56-1.79)	
alleles				
F[a]	180 (52.6)	188 (53.4)	1.00 (Ref)	0.84
f	162 (47.4)	164 (46.6)	1.03 (0.76-1.41)	
Bsm I				
genotypes				
bb	144 (84.2)	143 (81.2)	1.00 (Ref)	0.47
bB + BB	27 (15.8)	33 (18.8)	0.87 (0.49-1.53)	
alleles				
b	314 (91.8)	316 (89.8)	1.00 (Ref)	0.35
B	28 (8.2)	36 (10.2)	0.80 (0.48-1.33)	

[a] F, f, B, and b revealed C, T, A, and G, respectively. [b] Adjusted for sex and age by the logistic regression model.

VDR Fok I and *VDR Bsm* I polymorphisms between the cases and controls (for *VDR Fok* I: adjusted OR 1.03, 95% CI: 0.76-1.41; for *VDR Bsm* I: adjusted OR 0.80, 95% CI: 0.48-1.33).

As a polymorphism in the *VDR* start code, the frequencies of *VDR Fok* I genotype and allele between our control group in the present study and those of previous reports in different countries were also compared. Among the studies with Asian controls, mainly Chinese (*17*) and Japanese controls (*18*), the frequencies of F and f alleles were 47.2-60.5% and 39.5-52.8%. The allele distribution of *VDR Fok* I in this study had no significant difference from that found in Asians from other studies. Among the studies with Caucasian controls, the frequencies of F and f alleles were 65.6-68.8% and 31.2-34.4% (*19*). When compared with this study's controls, however, the Caucasian controls showed significantly higher allele F and lower allele f frequencies ($p < 0.05$). This result was in agreement with previous studies, which concluded the prevalence of the f allele of *VDR Fok* I polymorphism was 36% and 46% in controls of European and Asian descent, respectively (*20*). Furthermore, we compared the *VDR Fok* I and *Bsm* I allele frequency in this study's controls with HapMap (the International HapMap Project) genomes data in the National Institutes of Health Single Nucleotide Polymorphism database (dbSNP) (*http://www.ncbi.nlm.nih.gov/projects/SNP/*). It was shown that the distribution of the *VDR Fok* I allele frequency in this study's controls was in agreement with Chinese and European results. However, the frequency of F allele was higher in Japanese (73.3%) and in Sub-Saharan Africans (83.3%) than this study's controls (53.4%). The distribution of *VDR Bsm* I allele frequency in this study's controls was in agreement with Asians, but differed from Europeans and Sub-Saharan Africans. The frequency of B allele was significantly higher in Europeans (43.8%) and Sub-Saharan Africans (27.9%) than this study's controls (10.2%).

The present study, to our knowledge, is the first to investigate the potential correlation between the *VDR Fok* I and *VDR Bsm* I polymorphisms and NPC. In this pilot study, no significant association was found between *VDR Fok* I and *Bsm* I polymorphisms and the risk of development of NPC. The result implies that *VDR Fok* I and *VDR Bsm* I polymorphisms may not directly contribute to the susceptibility to NPC in the Chinese Han population.

Most of the biologic activities of $1_{\alpha},25(OH)_2D_3$ are mediated by a high-affinity receptor, VDR, which plays a role as a ligand-activated transcription factor. The main steps involved in the modulation of gene transcription by the *VDR* include binding of ligand, heterodimerization with retinoid X receptor (RXR), binding of the heterodimer to vitamin D receptor elements (VDREs), and recruitment of other nuclear proteins into the transcriptional pre-initiation complex. Therefore, genetic changes of the *VDR* gene could induce serious defects of gene activation, influencing calcium metabolism, cell proliferation, and immune function (*9*). Previous studies also implied that polymorphisms in the gene of *VDR* were associated with disease susceptibility, such as cancer, immune dysfunction and chronic disease. *VDR Fok* I polymorphism is a C-to-T transition within exon 2 of the *VDR* gene, defined by endonuclease Fok I. The *VDR Bsm* I genotype is at the 3' end of the VDR gene (intron 8), it has been proved that it does not lead to any change in either the transcribed mRNA or the translated protein. However, the two polymorphisms have been reported to be associated with malignancy (*12-14*), including breast cancer, prostate cancer, primary parathyroid tumors, and colorectal cancer. NPC is a malignancy arising from the epithelial cells lining the nasopharynx and it often occurs in Southern China and Southeast Asia. Our previous studies and other studies have shown that a range of cytokines (*7,8*), including *IL-1B*, *interleukin-8*, *interleukin-10*, and *transforming growth factor-b1* gene polymorphisms, may contribute to the development of NPC. Therefore, in combination with the correlation with *VDR* gene alteration and cancer risk, we postulate that *VDR Fok* I and *Bsm* I gene polymorphisms may modulate susceptibility to NPC. No significant association, however, was found between *VDR Fok* I and *Bsm* I polymorphisms and the risk of NPC in the current study.

In agreement with our findings, some studies have shown that the *VDR Fok* I polymorphism was not associated with breast cancer (*12*), colorectal adenomas (*19*), prostate cancer (*21*), and systemic lupus erythematosus (SLE) (*17*). In addition, some authors reported no associations were found between *VDR Bsm* I polymorphism in breast cancer (*22*), and prostate cancer (*23*). However, the association between

VDR polymorphisms and studied types of tumors is still controversial. Bodiwala *et al*. showed that the *VDR Fok*1 ff genotype was associated with an increased prostate cancer risk (OR = 2.91) (*24*). Hutchinson *et al*, figured that *VDR Fok*1 polymorphisms were correlated to an altered risk for malignant melanoma (MM) (p = 0.014) (*25*). Also Ingles *et al*. found that the Bb and BB genotypes had a 1.6-fold and 2.2-fold increased breast cancer risk than the bb genotype, respectively (OR = 1.6, OR = 2.2, respectively) (*26*). Furthermore, Habuchi *et al*, reported that the Bb and BB genotypes of *VDR Bsm*1 were associated with a significantly reduced risk for prostate cancer (OR = 3.31; 95% CI: 2.05-5.32) and with one-half the risk for benign prostate hyperplasia (BPH) (OR = 2.07; 95% CI: 1.33-3.22) when compared with the male controls (*27*).

These debatable results mentioned above may be due to various reasons, including a limited number of cases, or the analysis of different ethnic groups, or environment factors which the individuals were exposed to, or even gene-gene and gene-environment interactions. Most case-control studies reported no association with *VDR Fok*1 polymorphism and breast caner risk (*12*). However, when the samples were matched up to cohorts (1,234 cases, 1,676 controls), a significantly increased risk of breast cancer was observed (*28*). Chaimuangraj *et al*. focused on the Chinese Han population and suggested that variations in the distribution of *VDR Bsm* I in different ethnic groups might contribute to the racial difference in prostate cancer risk (*29*). Also there is a significant increase of prostate cancer risk found for African-Americans with the ff genotype but not for US Caucasians (*23*). Moreover, Michelle Guy found that when analyzed independently, *Fok* I was not associated with breast cancer risk ($p > 0.05$). However, when it was analyzed combined with *VDR Bsm1* and poly (A) polymorphism, *Fok* I did modulate the increased risk associated with the bb/LL genotype, and one or more F alleles together with the bb/LL genotype enhanced breast cancer risk (*12*). Furthermore, taking environmental factors into consideration, Li *et al*. concluded that the *VDR Fok*1 polymorphism is not an independent risk factor for MM, but it could modulate melanoma risk when it interacts with skin color ($p = 0.029$), moles ($p = 0.017$) and number of first-degree relatives with any cancer ($p = 0.013$) (*30*).

This study also has some limitations. One is that the detailed information on the survival from NPC is unavailable, which restricted our further analysis on the role of *VDR Fok* I and *Bsm* I in cancer prognosis. Another is that the association between *VDR Fok* I and *Bsm* I gene polymorphisms and NPC should be analyzed in different ethnic populations.

In conclusion, we did not find that *VDR Fok* I and *Bsm* I gene polymorphisms were associated with the risk of NPC in a Chinese Han population. Nevertheless, due to complex reasons, further studies will be needed to explore the complicated gene-gene interactions and gene-environmental interactions in the susceptibility to NPC, especially in ethnically disparate populations in cohort study samples.

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Study of KAP with regard to taking folic acid supplements and factors affecting the recommendation and prescription of those supplements among obstetricians and specialists in women's health in six provinces of Northern China, 2009

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Summary **Taking folic acid (FA) supplements reduces the risks of neural tube defects (NTDs) in early pregnancy. Obstetricians and specialists in women's health play important roles in promoting FA intake. However, surveys on their knowledge of, attitudes toward, and behavior regarding giving FA to pregnant women are limited. A cross-sectional study was conducted with 5,860 obstetricians and specialists in women's health using a self-administered questionnaire to collect information on participants' demographic characteristics and their knowledge of and attitudes toward and practices related to taking FA supplements. Chi-square analysis was used for rate comparison while logistic regression analysis was performed to predict influencing factors. For items on knowledge about FA and taking FA supplements the overall correct response rate was 60.3% (24,235/40,173). Questions about related practices and attitudes were correctly answered for the most part (more than 90%), but participants were less likely to follow the practice of prescribing FA tablets to women planning a pregnancy while working (77.2%). Statistical analysis indicated that the "Level of facility where the participant works" and "Rate of correct responses on a test of knowledge" were the main factors affecting participants' recommendation to take FA while "Job title", "Amount of professional work experience", and "Rate of correct responses on a test of knowledge" were the main factors affecting participants' prescription of FA. In conclusion, participants had a good deal of knowledge about NTDs and FA but the lack of some knowledge possibly led to the relatively low rate of correct behaviors. Therefore, educating obstetricians and specialists in women's health in this regard is crucial.**

Keywords: Folic acid, folate, neural tube defects, obstetrician

1. Introduction

Neural tube defects (NTDs) are some of the most common birth defects since they lead to miscarriages, fetal death, stillbirth, and permanent infant disability. The incidence of these defects is typically 2.3~2.5 per 1,000 births in China, but this figure is as high as 6.0~10.0 in some areas according to Birth Defects Monitoring (*1*). This group of congenital anomalies includes anencephaly, encephalocele, iniencephaly, meningocele, myelomeningocele, myeloschisis, lipomeningocele, and spina bifida.

Folic acid (FA) is a water-soluble vitamin that is closely associated with NTDs (*2*). FA is not a new drug but is an existing chemical that has been available since the 1950s. FA has been widely utilized to prevent folate deficiency (*3*), one of the most common vitamin

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deficiencies among women today. Research has shown that women who consume a low level of folate during pregnancy are at risk of poor pregnancy outcomes, including some birth defects such as NTDs (*4*). Previous studies confirmed that women of childbearing age should consume at least 0.4 mg of FA daily, especially before or during early pregnancy (*5,6*). This may not only reduce the risk of NTDs by 50-70% but may also prevent other major birth defects (*7*). A large prospective study conducted by American research group found a 64% lower risk of NTDs in the offspring of women using multivitamins during the first 6 weeks of pregnancy; the offspring of those who took multivitamins containing folic acid had a 73% lower risk, whereas the offspring of those who took multivitamins without folic acid had only a 7% lower risk (*8*). This epidemiological evidence was strengthened by a nonrandomized intervention study carried out in China (*9*). Periconceptional use of daily supplements containing 0.4 mg of folic acid led to a 79% reduction in the risk of NTDs in Northern China (with a higher baseline rate of NTDs) and a 41% reduction in the South (with a much lower baseline rate of NTDs). Consequently, since the early 1990s health authorities in many developed countries have recommended that all women planning pregnancy should consume additional dietary and supplementary FA preconceptionally (*10*). In 2006, the WHO released a guide on folate fortification to promote folic acid supplements worldwide (*11*). In 1993, the Chinese Ministry of Health required that all newly married women should take 0.4 mg/day of oral FA supplements from marriage until 3 months after pregnancy; for women living in cities, the schedule was changed to when women began planning their pregnancies. Despite these recommendations and national campaigns, preconceptional intake of additional FA remains very low in some areas of China. A previous survey indicated that the intake rate was 12.1% and only 4.5% when used periconceptionally in the northern areas of China, which had a high prevalence of NTDs in 2008 (*12*).

Obstetricians and specialists in women's health play significant roles in promoting FA intake. Their knowledge of and attitudes toward FA directly affect the use of FA supplements, in turn helping reduce the incidence of NTDs. In April, 2008, the Chinese Ministry of Health implemented its "Project on birth defect interventions in six provinces of Northern China". The project covered a total of 293 impoverished counties in the six provinces of Shanxi, Inner Mongolia, shaanxi, Gansu, Qinghai, and Xinjiang, all of which have a high incidence of NTDs. Northern China faces increased prevalence of NTDs, especially in poor regions. A previous study showed that the prevalence of NTDs in those six provinces was up to 16.0/10,000 births of at least 28 weeks of gestation in 2008 (*12*). Prevention and treatment of birth defects by steps such as taking FA supplements may be crucial to women in those areas.

In order to provide a scientific basis for enhancing intervention in the form of recommending or prescribing FA supplements, the current study investigated the knowledge, attitudes, and practices (KAP) of obstetricians and specialists in women's health with regard to the addition of small doses FA for childbearing women to prevent NTDs. This study also analyzed factors affecting the behaviors of these obstetricians and specialists in women's health.

2. Materials and Methods

2.1. *Participants*

A total of 5,860 obstetricians and specialists in women's health who were involved in county-level projects to prevent birth defects participated in this study. Twenty participants were selected from each of 293 poor counties in six provinces.

2.2. *Survey tools*

A self-administered questionnaire was designed based on a review of the literature. The questionnaire asked about *i*) the participants' demographic characteristics such as age, ethnicity, level of education, level of the facility where the participant works, department, job title, and work experience, *ii*) knowledge about taking FA supplements, such as knowledge about NTDs and FA, and taking FA supplements, *iii*) attitudes toward taking FA supplements, and *iv*) the routine practice of recommending FA supplements.

2.3. *Survey method*

A cross-sectional study was carried out using an anonymous self-administered questionnaire. This study was conducted with the participants' consent.

2.4. *Statistical analysis*

EpiData 3.02 (EpiData Association, Odense, Denmark) was used to develop a study database, and SPSS 16.0 (IBM, Armonk, NY, USA) was used for statistical analysis. Chi-square analysis was used for rate comparison and logistic regression analysis was performed to predict influencing factors. The level of statistical significance was less than 0.05 in this study.

3. Results

3.1. *Demographic characteristics*

All 5,860 obstetricians and specialists in women's health received a questionnaire; the response rate was 100%. Incorrectly completed questionnaires were eliminated,

resulting in 5,739 valid questionnaires (97.9%). The 5,739 participants had an average age of 35.6 ± 8.1 years, and most participants were ages 31-40 (44.0%) while few were under the age of 20 (0.4%). Most participants belonged to the Han ethnic group (80.1%). Subjects with an associate degree accounted for 43.6% of the participants, those with high school or secondary education accounted for 41.1%, and those with undergraduate or higher education accounted for 11.4%. The facility where the participant works was most often a district-level or county-level hospital (61.1%), followed by a township hospital (31.6%) or a municipal hospital (4.6%). In terms of their job titles, most participants were specialists (55.8%) although a few were senior-level or administrators (4.8%). Most participants had five or more years of professional work experience (75.2%).

3.2. *Survey of participants' KAP*

Seven questions about NTDs knowledge resulted in a total of 40,173 responses from the 5,739 participants. Of these, 24,235 were correct responses, indicating a correct response rate of 60.3% (24,235/40,173). In particular, awareness of "when NTDs occur" was least often answered correctly, that is, only 27.2% of participants responded correctly. Nine questions on knowledge about FA and taking FA supplements had 32,656 correct responses, resulting in an overall correct response rate of 63.2% (32,656/51,651). Only 34.1% participants knew that "women planning to become pregnant and pregnant women-to-be should both take FA supplements", while 37.5% knew that "taking 0.4 mg of FA each day meets the daily requirement for FA". With regard to participants' attitudes, more than 90% correctly answered questions on related practices and attitudes. However, fewer participants prescribed FA tablets to women planning to become pregnant while working (77.2%) (Table 1).

3.3. *Analysis of factors influencing behavior related to recommending/prescribing FA supplements*

Differences in age, level of education, the level of facility where the participant works, and job title led to different rates of following the practice of "usually recommending that women take FA supplements". Participants of the Han ethnicity were more like to follow the practice than were other ethnic groups; gynecologists/obstetricians were more like to follow the practice than were specialists in women's health. Participants with five years of professional working experience were more likely to answer correctly than those with one to five years; participants with a 60% overall correct response rate were more likely to correctly answer questions related to knowledge of taking FA supplements than those with a correct response rate of less than 60% ($p < 0.05$) (Table 2).

Differences in age, level of education, the level of facility where the participant works, and job title led to different rates of following the practice of "routinely prescribing FA tablets to women planning to become pregnant while working". Participants of the Han ethnicity were more like to follow the practice than were other ethnic groups; gynecologists/obstetricians were more like to follow the practice than were specialists in women's health. Participants with five years of

Table 1. Participants' KAP related to taking FA supplements

Index	Number of participants with correct responses	Correct response rate (%)
K: Knowledge of NTDs		
Definition of NTDs	5,477	95.4
When they typically occur	1,561	27.2
Types of NTDs	2,232	38.9
The most common type of NTDs	3,325	57.9
Prognosis for infants with NTDs	4,144	72.2
Definition of spina bifida	3,194	55.7
Parts of the body commonly affected by spina bifida	4,302	75.0
K: Knowledge about FA and taking FA supplements		
What is FA	5,041	87.8
Main source of human folate	4,133	72.0
Which women should take FA supplements	1,958	34.1
Types of deformity prevented by FA	5,138	89.5
Correct dose of FA for typical women of childbearing age	4,576	79.7
Correct dose of FA for women at risk of having an infant with NTDs	2,775	48.4
Correct way to take FA	4,913	85.6
Correct time to take FA	1,969	34.3
How to ensure a sufficient quality of FA each day	2,153	37.5
A: Attitudes toward taking FA supplements		
Pregnant women should take FA	5,553	96.8
Doctors need to discuss FA with women of childbearing age	5,556	96.8
Doctors should routinely prescribe FA tablets to women of childbearing age who plan to become pregnant	5,277	91.9
The importance of FA will be explained to every woman of childbearing age in the future	5,477	95.4
P: Practices with regard to taking FA supplements		
You usually recommend that women take FA while working	5,183	90.3
You routinely prescribe FA tablets to women planning to become pregnant while working	4,430	77.2

professional working experience were more likely to answer correctly than those with one to five years; participants with a 60% overall correct response rate were more likely to correctly answer questions related to knowledge of FA addition than those with a correct response rate of less than 60% ($p < 0.05$) (Table 3).

Variables with $p < 0.20$ in univariate analysis were subjected to multivariate logistic regression analysis; Table 4 shows the coding of variables. With an α, the significance level, of 0.05, the variables in the regression model of the practice of "usually recommending that women take FA supplements" were: ethnicity, level of education, the level of facility where the participant works, job title, professional working experience, and rate of correct responses on a test of knowledge. In conclusion, the higher the level of facility where the participant works, the more likely the participant is to recommend that women take

Table 2. Univariate analysis of participants' behavior of "usually recommending that women take FA supplements"

Variable	Groups of responses	Number of participants with correct behaviors	Rate of correct behaviors (%)	χ^2	*p*-value
Age	~ 20	23	95.8	1.464	0.691
	~ 30	1,446	89.9		
	~ 40	2,286	90.6		
	40 +	1,306	90.1		
Ethnicity	Han	4,183	91.0	17.85	< 0.05
	Other	917	86.8		
Level of education	Junior high school or lower	148	84.1	13.14	< 0.05
	Senior high school or secondary school	2,125	89.4		
	Junior college	2,279	91.1		
	Undergraduate or higher	603	91.8		
Level of facility where the participant works	Village/community	59	88.1	25.20	< 0.05
	Township	1,586	87.5		
	District and county	3,244	91.7		
	City	202	89.8		
	Province	39	90.7		
Job title	Specialist	2,885	89.1	11.55	< 0.05
	Senior specialist	2,021	91.7		
	Administrator	252	92.3		
Amount of professional work experience	One to five years	1,100	88.6	5.762	< 0.05
	Over five years	3,922	90.9		
Rate of correct responses on a test of knowledge	Less than 60%	1,603	82.8	188.4	< 0.05
	60% or higher	3,580	94.1		

Table 3. Univariate analysis of participants' behavior of "routinely prescribing FA tablets to women planning to become pregnant while working"

Variable	Groups of responses	Number of participants with correct behaviors	Rate of correct behaviors (%)	χ^2	*p*-value
Age	~ 20	21	87.5	4.287	0.232
	~ 30	1,227	76.3		
	~ 40	1,939	76.8		
	40 +	1,141	78.7		
Ethnicity	Han	3,580	77.9	9.423	< 0.05
	Other	777	73.7		
Level of education	Junior high school or lower	133	75.6	7.919	< 0.05
	Senior high school or secondary school	1,791	75.4		
	Junior college	1,958	78.3		
	Undergraduate or higher	521	79.3		
Level of facility where the participant works	Village/community	47	70.1	24.07	< 0.05
	Township	1,337	73.7		
	District and county	2,803	79.3		
	City	168	74.7		
	Province	35	81.4		
Job title	Specialist	2,390	74.6	31.13	< 0.05
	Senior specialist	1,781	80.8		
	Administrator	221	81.0		
Amount of professional work experience	One to five years	906	72.9	15.20	< 0.05
	Over five years	3,377	78.2		
Rate of correct responses on a test of knowledge	Less than 60%	3,078	80.9	89.80	< 0.05
	60% or higher	1,352	69.8		

FA supplements (OR < 1). Participants with a correct response rate higher than 60% were more likely to recommend FA than those with a rate below 60% (OR > 1) (Table 5).

With an α of 0.05, the variables in the regression model of the practice of "routinely prescribing FA tablet to women planning to become pregnant while working" were: ethnicity, level of education, the level of facility where the participant works, job title, professional working experience, and rate of correct responses on a test of knowledge. A higher job title and longer professional working experience resulted in a greater likelihood of routinely prescribing FA tablets to women planning to become pregnant (OR < 1). Participants with 60% or more correct responses on the test of knowledge were more likely to routinely prescribe FA tablets to women planning to become pregnant than were those with a correct response rate below 60% (OR > 1) (all $p < 0.05$) (Table 6).

4. Discussion

This study was the first attempt at a large sample study of Northern China, an area facing a high incidence of NTDs. With the help of medical personnel, this study sought to determine the KAP of obstetricians and specialists in women's health with regard to taking FA supplements in these provinces. The current results may provide valuable reference data for national interventions to reduce the incidence of NTDs and thus improve the country's health.

The Chinese Ministry of Health's "Project on birth defect interventions in six provinces of Northern China" was implemented in April 2008. The current

Table 4. Univariate analysis of participants' behavior of "usually recommending that women take FA supplements"

Variable	0	1	2	3	4
Behavior	True	False			
Age	~ 20	~ 30	~ 40	40 +	
Ethnicity	Han	Other			
Level of education	Junior high school or lower	Senior high school or secondary school	Junior college	Undergraduate or higher	
Level of facility where the participant works	Village/Community	Township	District and County	City	Province
Job title	Specialist	Senior specialist	Administrator		
Professional work experience	Over five years	One to five years			
Rate of correct responses on a test of knowledge	Above 60%	60% or lower			

Table 5. Multivariate logistic regression analysis of participants' behavior of "usually recommending that women take FA supplements"

Risk factors	B	S.E.	OR	95% CI for OR		*p*-value
				Lower	Upper	
Constant	–3.374	0.350	0.034			0.859
Ethnicity	0.198	0.111	1.219	0.981	1.515	0.075
Level of education	–0.041	0.069	0.960	0.838	1.098	0.549
Level of facility where the participant works	–0.180	0.082	0.836	0.711	0.981	< 0.05
Job title	–0.132	0.089	0.876	0.735	1.044	0.139
Amount of professional work experience	0.161	0.111	1.174	0.944	1.461	0.149
Rate of correct responses on a test of knowledge	1.169	0.096	3.218	2.664	3.886	< 0.05

Table 6. Multivariate logistic regression analysis of participants' behavior of "routinely prescribing FA tablets to women planning to become pregnant while working"

Risk factors	B	S.E.	OR	95% CI for OR		*p*-value
				Lower	Upper	
Constant	−1.735	0.244	0.176			0.986
Ethnicity	0.125	0.083	1.133	0.963	1.332	0.132
Level of education	−0.018	0.048	0.982	0.894	1.080	0.713
Level of facility where the participant works	−0.103	0.058	0.902	0.805	1.012	0.078
Job title	−0.193	0.063	0.824	0.729	0.932	< 0.05
Amount of professional work experience	0.201	0.079	1.223	1.047	1.429	< 0.05
Rate of correct responses on a test of knowledge	0.618	0.068	1.856	1.623	2.121	< 0.05

study should furnish a baseline. Since obstetricians and specialists in women's health are responsible for counseling and issuing prescriptions as part of efforts to prevent birth defects, their knowledge, level of education, and ability to serve are all relevant to the qualities of these efforts. Extremely significant is the fact that study participants included doctors who were involved in training to prevent birth defects in areas with a high incidence of NTDs. Results demonstrated that some participants were highly knowledgeable about NTDs and FA since over 80% knew that FA can be used to prevent NTDs and they knew how to correctly take FA tablets. This figure was higher than that in a 2005 survey of birth control specialists in Sichuan Province (*13*). Of the current participants, 69.7% knew of FA's preventive action, and 41.9% knew how to correctly take FA tablets. These results indicated that national projects to implement interventions in NTDs using FA over the past few years have greatly improved doctors' knowledge about FA and NTDs. This is especially true of doctors in regions with a high incidence of NTDs. However, there are gaps in knowledge in comparison to other countries. According to a survey of American healthcare providers from 2002 to 2003, 58% of subjects knew how to correctly give FA supplements to high-risk women (*14*). A survey in 2004 showed that 92.3% of sixty Canadian doctors correctly answered questions about FA (*15*). Another study in 2002 showed that awareness of FA among 202 Indian physicians was 92.1% (*16*). All of these results indicate that Chinese medical personnel in northern regions with a high incidence of NTDs still need to strive to close the gap in knowledge.

The current study showed that only 34.1% of all participants were aware of the need for women who can become or who are planning to become pregnant to take FA supplements, 34.3% knew the correct time to take FA, and 48.0% knew how to correctly give FA to women at risk of having infants with NTDs. Similar results were found in a 2008 survey in southern Israel that showed a lack of knowledge about the correct timing (12%) of folic acid preparations for average-risk women (*17*). Though most of the current participants were aware that FA has a role in preventing NTDs, their knowledge about some key points such as its timing and who needed to take the supplements was lacking. Therefore, medical personnel should be educated about the need to use FA to prevent NTDs at the proper time for women who can become or who are planning to become pregnant.

Although the vast majority of participants expressed willingness to discuss the importance of FA with women of childbearing age, the reality is that only 77.2% routinely prescribed FA tablets; this may due to a lack of knowledge such as "which women should take FA supplements" and the "correct time to take FA". The 2008 survey in Israel indicated that 70 of 81 physicians (94%) reported routinely recommending FA to their patients (*17*). Thus, efforts should be made to increase awareness regarding prevention of NTD's by taking FA supplements at the proper time. Similarly, efforts must be made to educate medical personnel about correct behaviors with regard to recommending/prescribing FA supplements.

Multivariate analysis suggested that doctors in higher level hospitals were more likely to receive training, making them more likely to recommend/prescribe FA supplements to women. Stricter working conditions also resulted in a higher rate of correct behaviors. Moreover, participants in higher positions tended to routinely prescribe FA tablets to women planning to become pregnant, which might be related to their greater amount of knowledge and working experience. Furthermore, participants with more than five years of working experience had a higher rate of correct behaviors than those with less than five years of experience, which also coincides with the results of a similar study in 2008 of 101 birth control information officers in Yunnan Province (*17*). Therefore, the higher rate of correct behaviors is presumably the result of combined elements of longer working experience, more experience, greater awareness of the need for relevant knowledge, and more opportunities to receive education and training. With regard to the two correctly followed practices, the number of participants who had a correct response rate of 60% on the knowledge test was respectively 3.218 and 1.856 times that of those who had a correct response rate below 60%. Clearly, the level of one's knowledge plays a critical role in deciding if one's behavior is appropriate; therefore, offering training in FA and NTDs to obstetricians and specialists in women's health is especially crucial.

In general, participants had a good deal of knowledge about NTDs and FA but the lack of some knowledge possibly led to the relatively low rate of correct behaviors. The four main factors that were strongly associated with the rate of correct behaviors were found to be the level of facility where the participant works, job title, professional work experience, and the correct response rate on a knowledge test. This study also suggested that training obstetricians and specialists in women's health in NTDs and FA should have a marked effect.

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Original Article

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The effects of instruction regarding sleep posture on the postural changes and sleep quality among middle-aged and elderly men: A preliminary study

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Summary **The purpose of this study was to examine whether instruction to sleep in a lateral posture prior to falling asleep could increase the frequency of instructed posture and sleep quality, as evaluated by sleep parameters and a questionnaire for subjective assessment of sleep. The participants were comprised of 8 middle-aged and elderly men who had an awareness of their habitual snoring during sleep. Data were gathered from observations of sleep posture, sleep polysomnography and a subjective sleep quality questionnaire. As a result of the instruction, the frequency of the instructed posture was significantly increased, and there were no significant effects on sleep parameters or the frequency of postural changes. The subjective sleep quality during the instructed sleep showed worse scores than free postural-sleep for all factors. Our findings suggest that the instructed sleep posture could be increased during sleep without substantially worsening the sleep parameters and the frequency of postural changes. Future studies will therefore be required to clarify the mechanism and the long-term effects of such instruction on sleep posture, including the influence on subjective sleep quality.**

Keywords: Instruction of sleep posture, postural change, sleep parameters, subjective sleep quality

1. Introduction

Body movements during sleeping are classified as minor movements and major movements based on the amplitude using the static charge method (*1*). Change in sleep posture, or rolling over, is included in the major movements, which are accompanied by a transfer of the center of gravity. It is reported that the type and frequency of major movements differ significantly from individual to individual (*1*).

Rolling over is defined as a Postural Change during Sleeping (PCS). PCS is considered to have a physiological function, such as enhancing blood circulation and avoiding or decreasing the pressure on certain areas of the body. It is thought to be possible to regulate body temperature and moisture in bed by the physiological function of anti-side diaphoresis and PCS (*2*). Furthermore, PCS converges just before Rapid Eye Movement (REM) and the latter part of the REM phase (*3*). PCS is considered to be an important factor for REM; PCS enhances the transition from the waking stage to the sleeping stage, and is related to the procession and sustaining mechanism of the sleep cycle (*4*,*5*). As a result, there have been numerous reports regarding the physiological effects and roles of PCS.

When PCS is accompanied by large movements of the trunk, head and pelvis, it sometimes causes an awakening reaction on the electroencephalogram (EEG), thus indicating the presence of an alpha wave and light sleep; however, the short term awareness between a few and ten seconds during sleep are not remembered. PCS is generally accomplished

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unconsciously, and the trunk direction is unconsciously decided. Therefore, fixing the body in a compulsory manner seems to be a reliable method when a client's PCS should be controlled for the treatment of certain diseases.

It is reported that the apnea-hypopnea-index among patients who have sleep apnea syndrome, especially obstructive sleep apnea, shows improvement in the lateral posture rather than the supine posture (*6*,*7*). Therefore, an increase in the rate of lateral sleep posture would help prevent an apnea event, and some studies have reported interventions for controlling sleep posture using physical methods (*8*-*10*). These studies reported that the parameters of a sleep polysomnograph between intervention nights and controlled nights showed no differences. However, the compulsory intervention in sleep posture could have an effect on the frequency of PCS and subjective sleep quality.

A previous study examined the time that participants sustained the instructed posture during sleep when the sleep posture was instructed prior to sleep (*11*). It was suggested that the instruction could influence sleep posture, and that the instruction would be the easiest way to control sleep posture for the participants who had a general ability to understand and communicate without the need for physical restraint or other compulsory methods. Furthermore, sleep posture could be improved without interfering with the various natural roles of PCS. However, the previous study was not able to clarify the effects of instruction of sleep posture on sleep quality. Therefore, the purposes of the present study were *i*) to clarify the instruction of sleep posture prior to sleep which could increase the rate of the instructed posture during sleep and *ii*) to clarify the effect of instructed posture toward sleep quality, including such factors as sleep parameters and subjective sleep quality.

2. Materials and Methods

2.1. *Participants*

The participants were recruited in a Silver Human Resources Center or by a snowball sampling method. The participants were 8 males aged 51 to 72 (mean ± S.D.: 63.1 ± 6.9, median: 64.5). They had awareness of snoring during sleep, had no treatment history for sleep disorders, and were not using medication that affected the central nervous system. The average body mass index (BMI) was 25.2 ± 4.1 kg/m^2. Four out of 8 participants were smokers (Table 1). We targeted middle-aged and elderly men because their sleep structures were more susceptible due to aging and sex (*12*), because it has been shown that aging is a risk factor for Sleep Disordered Breathing (SDB), and an increase in incident prevalence until 60 years of age was also reported (*13*). Sleep disorders among women were most likely accompanied by menopause (*14*) and the ratio of insomnia per capita among women who were aged 50-70 and older surpasses that of their male counterparts (*15*).

Table 1. Demographics and characteristics of participants

	Mean ± S.D. [range] or *n* (%) (*n* = 8)
Age (years)	63.1 ± 6.9 [51-72]
Height (m)	1.68 ± 0.09 [1.57-1.84]
Weight (kg)	71.0 ± 13.4 [54-95]
BMI (kg/m^2)*	25.2 ± 4.1 [21.1-34.1]
Current smoker	4 (50)
Drinking (days/week)	4 or more:5 (62.5), 0:3 (37.5)
Awareness of snoring	Often:7 (87.5), sometimes:1 (12.5)

* BMI: body mass index.

A full explanation of all procedures and possible outcomes was given to the participants, and written informed consent was obtained from each participant. This research was reviewed by the Research Ethics Committee of Saitama Prefectural University.

2.2. *Schedule and environment of the experiments*

Participants were evaluated during a consecutive 3 night experimental session. The authors developed a schedule that sustained the participants' habits, such as time for awakening, going to bed, meals and bathing. Participants arrived at the laboratory 3.5 h earlier than their usual schedule for going to bed, and polysomnography electrodes were attached after having dinner (3 h before going to bed). Participants were prohibited from consuming alcoholic and caffeinated beverages after they entered the laboratory. They answered the questionnaire about sleep quality after waking up at the scheduled time, and wore a wrist actigraph device (Micro-Mini Actigraph; Ambulatory Monitoring Inc., Ardsley, NY, USA). They were allowed to do anything they wanted except for taking naps and engaging in intense exercise during the daytime.

During the sleep period, an environmental control chamber (Tabai Espec, Tokyo, Japan) was used to maintain the level of temperature and humidity at levels based on the guidelines from the Healthcare Engineering Association of Japan (26.0°C, 50% RH) (*16*). Normal bedclothes were used, a mattress was put on the floor in the experimental room, and a cotton blanket was used for cover.

2.3. *Measurement items and procedures*

2.3.1. *Sleep posture*

Sleep posture was recorded with an infrared video camera (TK-N1100; Victor, Kanagawa, Japan) between

going to bed at night and rising the next morning. The first night was the acclimation night, and participants were informed to roll over freely. For the second and third nights, the subjects were randomly provided instructions for sleep posture using a cross-over method: some patients received instructions the second night, others received them the third night. During the instructed sleep (Instruction-S), the patients were asked to: 'Please sleep in a lateral position as much as possible, it doesn't matter which side' and 'Please keep in a lateral position as much as possible while sleeping'. Meanwhile, free postural-sleep (Free-S) was defined as when the participants slept in their preferred posture.

2.3.2. *Sleep polysomnography*

EEGs were read using a mono-polar C3, C4, and Fpz parts based on the Ten Twenty Electrode System of the International Federation (*17*) and recorded with a Digital Multiuse Electroencephalograph (SYNAFIT5000; NEC Digital Systems, Tokyo, Japan). Electrooculography (EOG) was performed for both eyes, electromyography (EMG) was monitored for the mentalis muscle (both sides) and electrocardiography (ECG) (led between the right shoulder and left subclavicular) was also recorded at the same time. Data were gathered from the time when the subject went to bed until arising the next morning.

2.3.3. *Subjective sleep quality and sleep habit*

The subjective assessment of sleep states was measured with the Oguri-Shirakawa-Azumi sleep survey sheet- Middle Age and Aged edition (OSA-MA edition) (*18*), and a sleep onset questionnaire (*19*). The OSA-MA edition was a short form, targeting middle-aged and elderly people, revised by Yamamoto *et al.*, and based on the OSA sleep survey sheet, second edition (*20*) to estimate the subjective sleep profile that was developed by Oguri *et al.* The OSA-MA edition was scored using 5 factors with 16 items and 4 scales. Those five factors were *1*) sleepiness on rising (4 items), *2*) initiation and maintenance of sleep (5 items), *3*) frequent dreaming (2 items), *4*) feeling refreshed (3 items) and *5*) sleep length (2 items). A sleep onset questionnaire, revised by Yamamoto *et al.*, was a sociological estimation of sleep onset from going to bed and remaining asleep. In this experiment, the 9 items that estimate a sleep onset profile in the questionnaire were used because instructions just before sleep onset could possibly affect the sleep onset profile.

Participants confirmed that they had not traveled anywhere with a time difference of more than 5 h and had not engaged in night shift work within one month prior to the study. Their usual sleep and wake patterns were recorded using the wrist actigraph while they had worn the device on their non-dominant hand beginning one week before the experiment and throughout the experimental period. Participants responded to a Sleep Health Risk Index (SHRI) (*21*) and a questionnaire about habitual sleep posture that was originally developed by the authors. The SHRI, developed by Shirakawa *et al.*, aimed at clarifying sleep health risk by categorizing the degrees of risk for sleep health into 5 factors and scoring them (*21*). Another questionnaire about the awareness of posture during sleep was originally developed by the authors. It asked them about their awareness of their usual sleep postures of sleep onset and during sleep, then asked them why they slept in that posture.

The participants' awake states during the experimental night were measured using the wrist actigraph. Participants were asked about their awareness of posture during sleeping, such as sleep posture at sleep onset on each experimental night, and about the sleep posture that they were aware of most frequently during sleep.

2.4. *Analysis*

2.4.1. *Sleep posture and PCS*

Sleep postures recorded on video-tape were categorized visually in the following 4 directions based on the scapulas' direction: if participants slept on their back, it was defined as *i*) supine when the angle between the acromions and the mattress was between 0 to 45 degrees, *ii*) lateral sleep posture when the angle was between 45 to 90 degrees, if participants slept on stomachs, it was defined as *iii*) a lateral sleep posture when the angle was between 45 to 90 degree, and *iv*) prone when and the angle was between 0 to 45 degree (Figure 1). The categorization was done by one of the authors. The obtained data were analyzed every second and the duration of sustained posture was accumulated. The frequency of PCS was counted when a certain posture was sustained for more than 10 sec. Sleeping posture was analyzed for the whole night and it was divided into first half and second half sleep phases and the phases were compared.

2.4.2. *Sleep polysomnography*

Data were analyzed every 30 sec based on the International Classification of Sleep Process (Stage 1, 2, 3, 4, REM, Wake stage) (*22*). The first nights' data were excluded from the whole 24 nights' data of sleep polysomnography because of first night effects. Data from three participants was excluded from the analysis, because the data detection was incomplete or impossible to analyze. Therefore, data from a total of 10 nights were analyzed.

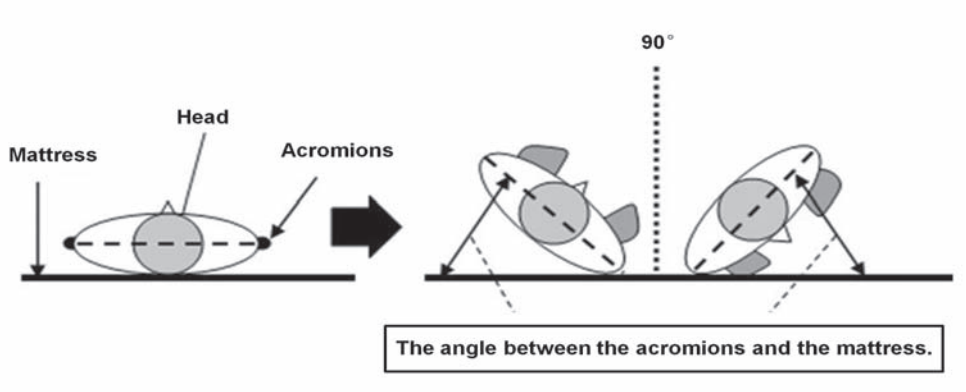

Figure 1. The categorization of sleep posture. The scapulas' direction: the angle between the acromions and the mattress. 0-45 degree, supine or prone; 45-90 degree, right or left lateral.

2.4.3. *Subjective sleep quality and sleep habit*

The OSA-MA edition was plotted in an MS-Excel sheet for converting sleep profile scores, and the results were analyzed based on the scores for 5 factors. In this process, high scores were judged as good sleep quality. Standard rating scales to estimate sleep onset were utilized as a sleep onset questionnaire and high scores were judged as good sleep onset quality.

Data that was consecutively recorded as activity level every 1 min with the wrist acitgraph were analyzed using a statistical software program with a specific interface, and the results were divided into sleep and awake phases based on the method reported by Cole *et al.* (*23*).

2.4.4. *Statistical analysis*

The SPSS 11.0J for Windows software package (SPSS Japan Inc., Tokyo, Japan) was used as a statistical tool. After excluding the first night data, Instruction-S and Free-S were compared. The paired *t*-test was used for comparisons of the sleep parameters. The Wilcoxon signed-rank sum test was used for the comparison of position rates, frequencies of PCS and subjective sleep quality data. Spearman's rank correlation coefficients were calculated to analyze the relationship of various parameters with the frequencies of PCS. The level of significance was set at $p < 0.05$.

3. Results

3.1. *Instructions and sleep postures*

The duration of each posture was calculated each night (mean ± S.D.). As a result, the supine position was used 64.9 ± 21.4% of the time, and the lateral posture accounted for 35.1 ± 21.4% of the first night, and for Free-S, the supine position was used 56.0 ± 18.2% and the lateral posture was used 43.7 ± 18.2% of the time. For Instruction-S, the supine position was used 14.2 ± 9.0% and lateral posture was used 85.8 ± 9.0% of the time. The prone posture was only used for 11 min in one participant's Free-S. The duration of the lateral posture thus increased during Instruction-S compared to Free-S among all 8 participants. The increase was 42.1 ± 12.5% (mean ± S.D.), which was a significant difference ($p = 0.012$). Figure 2 shows the rate of lateral posture after receiving instructions. The filled circle and the solid line represents the rate of lateral posture in 5 men whose polysomnographs were analyzed, and the open circle and the dotted line represents 3 men who were excluded.

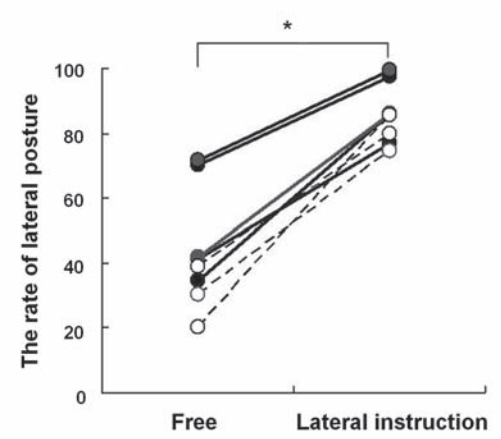

Figure 2. The rate of lateral posture [%] according to the presence of instructions; the change in each subject. The filled circle and the solid line represented the rate of lateral posture in 5 men whose polysomnographs were analyzed, and the open circle and the dotted line represented 3 men who were excluded. Wilcoxon signed-rank sum test; * $p < 0.05$.

Supine and lateral postures were compared based on sleep phases and whether instructions were given to the subjects. The duration (mean ± S.D.) of sleep postures between the first and second halves of sleep phases were compared. As a result, the supine position was used during the first half 61.0 ± 14.9% of the time, while it was used 51.2 ± 28.1% in the second half. The lateral posture during the first half was used 38.4 ± 14.8% of the time, and was used 48.8 ± 28.1% of the time when no instructions were given. Meanwhile, the supine position was used 12.4 ± 8.2% of the time and

16.5 ± 15.2% of the time in the first and second halves, while the lateral posture was used 87.6 ± 8.2% and 83.5 ± 15.2% of the time when subjects were instructed to sleep in a lateral position. The rates between the halves were not significantly different, regardless of whether or not instructions had been given.

3.2. *Instructions for sleep posture and the frequency of PCS*

The frequency of PCS (mean ± S.D.) per hour was analyzed separately when the instructions were given and not given. We observed that the frequency of PCS for Free-S was 2.2 ± 2.0 times/h while the frequency of PCS with Instruction-S was 2.2 ± 1.6 times/h. There were no statistically significant differences between the frequency of PCS with and without instructions. However, there were wide variations among participants during the first night, with a PCS frequency ranging from 0.4 times/h to 6.1 times/h, and there was a significant correlation between the frequency of PCS with and without instructions (r = 0.862, p = 0.006).

The frequency of PCS was also compared between the first and the second halves of sleep phases (mean ± S.D.). The frequency of Free-S for the first half was 1.9 ± 2.0 times/h, the frequency of Free-S for the second half was 2.4 ± 2.2 times/h, the frequency with Instruction-S for the first half was 1.8 ± 1.3 times/h and the frequency with Instruction-S for the second half was 2.7 ± 2.3 times/h. There were no statistically significant differences between the frequency of PCS during the first and second halves of sleep, regardless of whether instructions were given.

3.3. *Sleep postures and sleep parameters*

The rate of lateral posture during sleep in 5 men whose polysomnographs were analyzed and 3 men who were excluded were compared. No statistically significant difference was observed between the groups in terms of Instruction-S (p = 0.13) and Free-S (p = 0.06).

Table 2 shows each sleep parameter compared with and without instructions. There were no statistically significant differences between the parameters among the participants who slept with and without instructions. Figure 3 shows an example of sleep stages and postural changes that were extracted from a participant's final nights of Free-S (Figure 3a) and Instruction-S (Figure 3b). The participant went to bed with the instructed posture, and moved on to the third and fourth stages still sustaining the instructed posture.

The rate of sleep stages such as the first, second, third and fourth (Slow Wave Sleep: SWS), and REM sleep were analyzed with the exclusion of the times for not being in bed and being awake. The rates of each sleep stage were not significantly different between the Instruction-S and Free-S sleep. The rates of each sleep stage were compared for supine and lateral postures on each experimental night. There were no statistically significant differences between the rates of sleep stages with supine and lateral postures of Free-S, while the rates of SWS for the lateral posture significantly increased compared to the supine position (p = 0.04) when subjects were part of the Instruction-S group (Table 3).

3.4. *Subjective sleep quality and sleep habits*

Activities and rest in the daytime within the experimental terms were also recorded by the wrist actigraph. The length of activities in the daytime for sleep without instruction (mean ± S.D.) was 214.5 ± 25.3 min, and was 215.5 ± 27.0 min for Instruction-S, and the length of rest in the daytime for Free-S was 21.4 ± 35.9 min, while that for Instruction-S was 19.0 ± 24.9 min.

Scores of the OSA-MA edition were calculated separately for five factors. A higher score indicates a better quality of sleep. These scores were compared between sleep with and without instructions. The average scores for Instruction-S were lower than that for Free-S for all factors. The data of 5 men whose

Table 2. Comparison of various nocturnal sleep parameters in the presence of instructions

Parameter	Free[a]	Instruction[a]	p-value[†]
Time In Bed (min)	492.8 (14.5)	492.4 (13.9)	0.74
Total Sleep Time (min)	406.1 (46.0)	412.5 (25.9)	0.67
Waking After Sleep Onset (min)	74.2 (49.1)	61.1 (30.8)	0.41
Sleep Latency (min)	11.4 (7.3)	17.9 (14.7)	0.25
Sleep Latency to Stage 2 (min)	1.0 (2.0)	1.4 (2.0)	0.10
Sleep Latency to REM (min)	74.1 (41.9)	69.3 (34.9)	0.81
Sleep Period Time	480.3 (14.0)	473.6 (12.2)	0.29
Sleep Efficiency (%)	82.5 (10.6)	3.9 (7.3)	0.66
% Awake	15.4 (10.1)	12.8 (6.4)	0.43
% of Stage 1	7.7 (4.3)	7.7 (4.7)	0.99
% of Stage 2	45.8 (15.0)	44.8 (13.3)	0.70
% of SWS	13.2 (12.4)	17.1 (10.4)	0.29
% of REM	17.9 (5.3)	17.6 (7.6)	0.91

All values are the means (S.D.). [a] n = 5 for each group; [†] paired t-test. SWS, slow wave sleep (stage 3 and 4); REM, rapid eye movement.

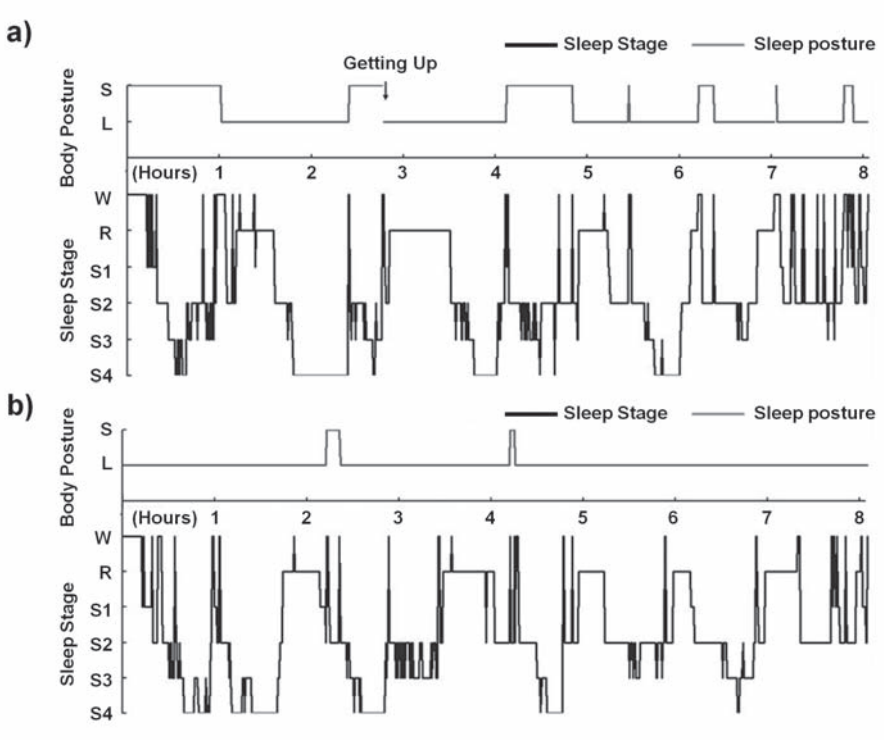

Figure 3. Nocturnal sleep stages and body posture in Free-S and Instruction-S. (a) Free-S: The free postural sleep: the participants slept in their preferred posture. **(b)** Instruction-S: The instructed sleep: the participants were asked to sleep in a lateral position as much as possible. Abbreviations: S, supine; L, lateral; W, awake; R, rapid eye movement; S1-4, stages 1-4, respectively.

Table 3. The rate of sleep stages according to a position in the presence of instructions

Sleep stage[b]	Free[a]			Instruction[a]		
	Supine	Lateral	*p*-value[†]	Supine	Lateral	*p*-value[†]
% Awake	16.5 (9.7)	14.3 (9.4)	0.14	29.2 (19.5)	10.7 (5.9)	0.15
% of Stage 1	7.7 (4.4)	7.8 (4.3)	0.91	10.6 (6.5)	6.8 (5.2)	0.48
% of Stage 2	52.9 (18.7)	46.4 (14.6)	0.17	30.6 (24.3)	46.9 (13.9)	0.36
% of SWS	14.2 (12.8)	13.3 (12.5)	0.18	1.6 (3.7)	19.2 (11.2)	0.03*
% of REM	8.6 (12.1)	18.2 (5.4)	0.08	28.1 (21.0)	16.5 (7.0)	0.36

All values are the means (S.D.). [a] $n = 5$ for each group; [b] Sleep stages except for awake time; [†] paired *t*-test; *$p < 0.05$. SWS, slow wave sleep (stage 3 and 4); REM, rapid eye movement.

Table 4. Evaluation of subjective sleep quality in the presence of instructions

Factor[a]	Free[b]	Instruction[b]	*p*-value[†]
I Sleepiness on rising	19.5 (3.5)	17.3 (4.9)	0.36
II Initiation and maintenance of sleep	21.8 (3.5)	17.0 (5.1)	0.06
III Frequent dreaming	25.5 (5.9)	19.8 (8.4)	0.04
IV Feeling of refreshment	18.3 (4.1)	16.8 (3.1)	0.22
V Sleep length	21.1 (4.9)	20.6 (6.0)	1.00

All values are the mean (S.D.).[a] The Oguri-Shirakawa-Azumi sleep survey sheet (MA edition) are classified under 5 factors (I-V), A higher score indicates a better quality of sleep; [b] $n = 8$ for each group; [†]Wilcoxon signed-rank sum test.

polysomnography results were analyzed also showed the same tendency. The factor of frequent dreaming for Instruction-S was significantly lower than for Free-S (Table 4). In terms of scores in the sleep onset questionnaire, sleep in the Free-S group was 23.5 ± 7.6, while that for Instruction-S was 19.7 ± 5.7, and there were no statistically significant differences between these scores.

The postures for habitual sleep onset and the postures that participants were aware of most frequently during sleep were analyzed according to participants' awareness of postures during sleeping. In total, 4 participants out of 8 reported using the supine position, 3 the lateral posture, and one the supine or lateral posture, in terms of their postures for habitual sleep onset. Meanwhile, 7 participants reported the supine and one reported the lateral posture in terms of their awareness of the most frequent posture while sleeping on the experimental nights when the instructions were not given. One out of 8 participants' awareness differed from the recorded data. In contrast, 7 participants reported the lateral posture and one reported the supine position during the experimental nights when Instruction-S was evaluated. The participants whose awareness differed from recorded data were again one

out of 8, and it was the same subject who had differed from the experimental nights when the instructions were not given.

The participants' feedback about what affected their habitual postures during sleeping was analyzed based on voluntary written reasons on the questionnaire. As a result, five out of 8 participants wrote that it was habit and one wrote that it was for the prevention of snoring.

4. Discussion

The major contribution of this study was to clarify that providing instruction on sleep postures could truly affect actual sleep postures, even though the participants were not trained. Moreover, the instructions increased the length of the instructed posture compared to the length of sustained lateral sleep posture without instructions. Furthermore, the instructions did not substantially affect the sleep parameters and the frequency of PCS. Recently, positional therapy, which is designed to minimize supine sleep, has become an important method in the successful management of stroke patients (*24*,*25*). This result suggested that such instructions represent a simple intervention method for improving sleep posture.

Miki *et al.* disciplined seven male OSA patients' sleep postures prior to their experiments, and gave an instruction about sleep posture on experimental nights (*26*). If patients changed position from the instructed sleep posture, the examiners corrected the sleep posture to the instructed posture without arousal. As a result, they concluded that the lateral position improved the apnea symptoms without inducing any significant difference in sleep parameters (*26*). However, they did not refer to the degree of increase in terms of the instructed posture or the influence on PCS and subjective sleep quality. We were apprehensive about possibly influencing sleep quality by such an intervention. The present study was therefore an intervention that only provides instructions about sleep postures given prior to going to bed, without intervention during sleep. However, the rate of the instructed posture during the experimental night's sleep was significantly increased. The participants' sleep parameters of Free-S in this study were similar to those of healthy men reported in a previous meta-analysis study (*12*). There were no significant differences between Instruction-S and Free-S in terms of the sleep latency, awakening after sleep onset, sleep efficiency, or the rate of the different stages. Furthermore, the rates of SWS for the lateral posture significantly increased compared to the supine position using Instruction-S. Therefore, when the participants were instructed to sleep in the lateral posture, they were still able to move on to deep sleep.

The frequency of PCS in our study correlated within individuals, but there were no statistically significant differences between the frequency of PCS during Instruction-S and Free-S. The rates of SWS in the second half of the total sleep time have been reported to decrease compared to the first half, while the frequency of PCS has been reported to increase with the repetition of a sleep cycle (*3*). Therefore, the postural changes were compared in the first and second halves of the sleep phases. The average frequency of PCS increased in the second half compared to the first half, regardless of whether participants received instructions; however, there were no statistically significant differences. For example, there is the tennis ball technique (TBT), which is a method to put a tennis ball under one's back during sleep, and this helps the patients to maintain a lateral posture (*27*). However, long-term patient compliance with TBT has been suggested to be poor (*28*). As a result, one potential disadvantage of TBT may be related to the fact that PCS is disturbed.

On the other hand, the participants were able to sleep in both lateral sleep postures when instructions were clearly provided in this study, and no disturbance in the participants' postural change was observed. Accordingly, because no difference in the frequency of PCS was observed, the physiological functions of PCS were therefore not suggested to be strongly influenced by the instruction provided in this study.

The subjective sleep quality with Instruction-S showed lower average scores than Free-S in all factors. The factor of 'Frequent dreaming' showed a statistically significant difference. The subjective sleep quality may have been worse in the Instruction-S group. This may have been because the subjects might have been aware of their sleep posture, and underwent a psychological load due to instruction of a posture that they did not like, although this was not proven. Of note, many participants responded that their habits influenced their sleep postures. This may have been related to the reason why 7 out of 8 participants were able to recognize their actual sleep postures, no matter whether instructions were given or not. However, so far, no studies have shown any concrete evidence regarding the mechanism of awareness of humans' sleep posture. In our previous study, correlations were observed between the body positions that participants chose when they fell asleep and in the actual head positions that were observed during sleep, even though there were some individual differentiations (*29*). It suggested that participants would be aware of sleep postures. Although this study could not address the mechanism of the increase in instructed posture, this indicates that the instructions given to participants who sleep in unusual postures affected their subjective sleep quality. We should also be aware of the possibility that a worsening of the subjective sleep quality may occur after participants are given instruction on sleep posture, in particular after the first instruction.

This study has several limitations. First, we only

investigated a small number of participants comprising middle-aged and elderly men. Therefore, this study was not able to clarify any effects regarding gender and age differences. This study also did not clarify the mechanism or the long-term effect of instructions on sleep posture, because the experimental term was only 2 nights. In addition, although the participants in this study had an awareness of their habitual snoring during sleep, this study was not able to elucidate any relationship between respiratory function of the participants and sleep quality. However, the results of this study suggest that giving instructions regarding sleep posture could provide a simple, noninvasive, and effective method for improving sleep posture.

Further studies are therefore required to recognize these limitations, and to clarify the mechanism and long-term effectiveness of such instruction on sleep posture, including influence on subjective sleep quality. Furthermore, it is necessary for the instruction of sleep posture to be evaluated in regard to whether it can actually improve respiratory function in obstructive sleep apnea patients.

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Original Article

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Gonadotropin-releasing hormone-agonist induces apoptosis of human granulosa-luteal cells *via* caspase-8, -9 and -3, and poly-(ADP-ribose)-polymerase cleavage

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Summary **Gonadotropin-releasing hormone-agonist (GnRH-Ag) used in controlled ovarian hyperstimulation (COH) for *in vitro* fertilization and embryo transfer (IVF-ET) has been known to directly affect apoptosis of human ovarian cells, but its mechanism is not clearly understood. Therefore, the purpose of the present study was to investigate whether caspase-8, -9, and -3 activation and poly-(ADP-ribose)-polymerase (PARP) cleavage are involved in the mechanism by which GnRH-Ag induces apoptosis in human granulosa-luteal cells. The prospective study was conducted in the research institute and clinical fertility center of university hospital. Human granulosa-luteal cells collected from IVF-ET patients were cultured and treated with 10^{-6} M GnRH-Ag or saline as a control. To access apoptosis in the cells, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end-labeling (TUNEL) assay and DNA fragmentation analysis were preformed 24 h after treatment. Activity of caspase-8, -9, and -3 in the cells was examined using a fluorogenic substrate. Caspase-8, -9, and -3 activation and PARP cleavage were analyzed by Western blot. DNA fragmentation in the cells increased at concentrations over 10^{-6} M GnRH-Ag. In TUNEL assays, the rate of apoptotic cell formation in GnRH-Ag treatment increased significantly compared with that of saline treatment ($p < 0.05$). The activity of caspase-8, -9 and, -3 investigated using a fluorogenic substrate increased only in the apoptotic cells. In Western blot analysis, cells treated with GnRH-Ag revealed an increase in active forms of caspase-8, -9, and -3 compared with saline treatment. In addition, cleavage of PARP also increased in cells treated with GnRH-Ag. These results suggest that activation of caspase-8, -9, and -3 and cleavage of PARP might be involved in apoptosis of human granulosa-luteal cells induced by GnRH-Ag.**

Keywords: Apoptosis, caspase, human granulosa-luteal cells, gonadotropin-releasing hormone-agonist, poly-(ADP-ribose)-polymerase

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a 10-amino acid protein synthesized in the hypothalamus that stimulates luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion *via* a connection with a receptor in the pituitary gland. This gonadotropin affects the gonads and associated organs (*1*). GnRH affects the ovary directly without involving the pituitary gland; specifically, GnRH influences physiologic changes in ovarian granulosa cells, ovarian growth, and ovulation (*2*,*3*). Because the half-life of GnRH secreted in hypothalamus is very short and GnRH is not detected in serum, the action of GnRH in the ovary was thought to be caused by GnRH synthesized within the ovary (*4*). Indeed, after the receptor for GnRH was identified in the human ovary, there have been studies focusing on the direct action of GnRH on the ovary (*5*,*6*).

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Specifically, there have also been active advances regarding the apoptotic action of GnRH on the ovary, as GnRH has been shown to directly induce apoptosis of human ovarian cells (*7*,*8*). GnRH has been reported to increase the expression of the Bax gene associated with apoptosis in luteal cells, but decrease the expression of the Bcl-xL gene associated with growth. Local inhibition of vascular endothelial growth factor (VEGF)-A activity appears to produce an increase in ovarian apoptosis through an imbalance among the BCL2 family members, thus leading a larger number of follicles to atresia (*9*,*10*). In addition, GnRH is known to be associated with a decrease in nitric oxide (NO), a known growth factor in luteal cells, but the signal transduction mechanism has not been clearly elucidated (*11*).

Generally, the mechanism of apoptosis involves the signal transduction process by which intracellular protein is degraded by a proteinase (caspase) and many kinds of caspases have been reported to be associated with apoptosis (*12*). Caspase-8 is activated by cell apoptosis induction materials, such as tumor necrosis factor (TNF)-α or Fas ligand, and activated caspase-8 activates other caspases which results in cellular apoptosis (*13*,*14*). During apoptosis, cytochrome c is released through a channel which exists in the mitochondrial membrane and is regulated by Bcl-2 proteins making up the channel. The released cytochrome c connects with Apaf-1, caspase-9, and dATP, and then activates caspase-9 (*15*,*16*). Activated caspase-9 activates caspase-3 which results in cellular apoptosis (*17*). As a result, activated caspase-3 activates poly (ADP-ribose) polymerase (PARP), which results in cellular apoptosis by inducing DNA fragmentation and nuclear condensation (*18*).

The purpose of the current study was to determine if apoptosis of human granulosa-luteal cells by GnRH-agonist (Ag) is caused by the above mechanism and to generate basic data needed for clinical use of GnRH-Ag. For this, cultured human granulosa-luteal cells were treated with different doses of GnRH-Ag and apoptosis was detected by DNA fragmentation and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end-labeling (TUNEL) method. In addition, to determine which caspases this apoptosis is associated with, we evaluated intracellular activity using a fluorescent substrate for caspases-8, -9, and -3 (*19*) and confirmed activated protein caspases-8, -9, and -3 and PARP by Western blot. As a result, the objective of this study was to elucidate the process of cellular apoptosis in human granulosa-luteal cells by GnRH-Ag.

2. Materials and Methods

2.1. *Obtaining human granulosa-luteal cells*

Human granulosa-luteal cells were obtained during ovum aspiration of patients undergoing *in vitro* fertilization (IVF). The use of human granulose-luteal cells was approved by the Clinical Committee of Life Science Institute in the Department of Obstetrics and Gynecology of Eulji Medical Center of Eulji University. All patients gave informed consent. All patients underwent the same ovarian stimulation protocol. Ovarian stimulation was achieved with a combination of pure FSH (Metrodin; Serono, Aubonne, Switzerland) and human menopausal gonadotropin (hMG) (Merional; IBSA, Lugano, Switzerland) in a step down fashion without a GnRH-Ag long protocol. Human chorionic gonadotrophin (hCG) (Profasi, 10,000 IU; Serono, Aubonne, Switzerland) was administered IM when at least 2 follicles reached an average diameter of 17 mm. Transvaginal oocyte retrieval (Medison 128; Medison Co., Seoul, Korea) was performed 35-36 h after hCG administration. The aspirated follicular fluid was transferred to culture dishes. The oocytes obtained under microscopy were analyzed and transferred to culture media. The granulosa-luteal cells existing within follicular fluid were obtained and transferred to culture media. To remove red blood cells, cultured fluid (1 mL) containing granulosa-luteal cells was layered on a 3 mL Percoll gradient and centrifuged for 20 min at 300 × g. After centrifugation, the granulosa-luteal cells were within the middle layer and the red blood cells had sedimented to the bottom.

The granulosa-luteal cells were washed 3 times with culture media and transferred to culture media with 0.1% collagenase (Sigma, St. Louis, MO, USA). After 30 min in culture media at 37°C, the cell clusters were separated into single cells by repetitive aspiration and expulsion through a 28G needle. The cells were counted on a hemocytometer. To determine viability, cells were stained with trypan blue. We used cells with a viability > 70% for culture at 100,000 cells per mL.

2.2. *Culture of granulosa-luteal cells*

Granulosa-luteal cells were placed in 24-well culture plates (Nunc, Roskilde, Denmark) at 100,000 cells per well; the cells were cultured in medium at 37°C with 95% air + 5% CO_2 in 100% humidity. The culture medium was Dulbecco's modified Eagle medium (dMEM; GIBCO BRL, Roskiville, NY, USA), 10% fetal bovine serum (FBS; GIBCO BRL), 2 mM L-glutamine (GIBCO BRL), 100 U/mL penicillin (GIBCO BRL), and 100 μg/mL of streptomycin (GIBCO BRL) were added.

Granulosa-luteal cells were transferred to 24-well culture plates and cultured for 24 h. The cells attached to the bottom of the plate were confirmed and the culture medium was changed. Cultured granulosa-luteal cells were treated with 10^{-6} M GnRH-Ag (Sigma), or normal saline as a control. After culture for 24 h, the cells were analyzed for cellular apoptosis and apoptosis-associated proteins.

2.3. *Analysis of DNA fragmentation to identify apoptosis*

Grind buffer (0.2 mL) was added to the granulosa-luteal cells, they were ground with a tissue grinder, and added to 12.5 μL of 10% SDS at 65°C. After 30 min, 35 μL of 8 M potassium acetate was added and mixed. Over a 60 min time interval, proteins were precipitated in ice at 4°C, and centrifuged at 5,000 × g for 10 min. The upper layer was transferred to a microcentrifuge test tube. After an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) was added, DNA was obtained. The same amount of chloroform/isoamyl alcohol (24:1, v/v) was added, and the DNA was re-obtained.

The upper layer fluid was transferred to a microcentrifuge test tube and 100% ethanol (2.5× volume) at 0°C was added, and then precipitated at −70°C in an ultra-low temperature refrigerator for > 1 h. DNA was obtained after centrifugation at 14,000 × g for 30 min at 4°C. The sediment was dissolved in 50 μL of 1× TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0); 1 μL of DNase-free RNase (500 μg/mL; Boehringer-Mannheim, Indianapolis, IN, USA) was added for 60 min at 37°C.

The DNA was extracted with the same amount of phenol/chloroform/isoamyl alcohol. After extraction, the DNA was re-extracted with the same amount of chloroform/isoamyl alcohol. After collecting the upper layer fluid, DNA was placed in 3 M sodium acetate (0.1 × volume) and 100% ethanol (2.5× volume) at 0°C, then at −70°C in a low-temperature freezer for > 60 min. The suspension was centrifuged at 14,000 × g for 30 min at 4°C, and was washed and dried in 80% ethanol (0.2 mL) at 0°C. We dissolved a compression determinant in distilled water (25 μL) at −20°C after measuring the quantity of DNA at 260 nm. A 5% agarose gel was loaded with DNA (5 μg) with TBE solution in running buffer, and stained with ethidium bromide after 3 h electrophoresis at 50 V, and observed in an ultraviolet ray transilluminator.

2.4. *TUNEL dyeing for determining cellular apoptosis*

Twenty-four hours after culture, apoptosis was detected using an *in situ* apoptosis detection kit (ApopTag; Intergen Co., Purchase, NY, USA) after fixation by TUNEL in the cultured granulosa-luteal cells. Granulosa-luteal cells were attached to a 24-well culture plate. Four percent neutral buffered formalin (500 μL) was placed in each well and the cells were fixed for 10 min and washed in Tris buffer. First, an equilibration buffer in an ApopTag kit was used for 5 min and after terminal deoxynucleotidyl transferase (TdT) enzyme was added, the response was observed for 90 min at 37°C. To stop the response, stop buffer was added for 10 min at room temperature, and samples were washed three times with Tris buffer. Anti-digoxigenin antibody with Texas red was applied for 30 min at 37°C, samples were washed with Tris buffer, and dyed secondarily with Hoechst 33258. After mounting with fluorescence mounting solution, we evaluated apoptosis in the cultured human granulosa-luteal cells using fluorescence microscopy. After the cells stained by TUNEL assay were counted, the ratio of the total cells was analyzed by staining with Hoechst 33258. Using an original magnification of 200×, the total number of cell nuclei and the number of TUNEL-positive cell nuclei were counted 5 times in randomly chosen fields on each treated or control coverslip. For quantitative analysis of apoptosis, we counted 300-500 cells for each slide. These counts were repeated five times in other experiments.

2.5. *Caspase-8 , -9, and -3 activity assay using fluorescent substrates*

Caspase activity in the cultured granulosa cells was assessed using the fluorescent substrate for each caspase (*19*). The cultured cells were washed in PBS containing the fluorescent substrates for caspases and incubated for 3 h at 37°C. After dyeing nuclei with Hoechst 33258, the cells were observed under fluorescent microscopy. Fluorescent substrates for activated caspase-8, -9, and -3 exhibited green fluorescence using fluorescent microscopy.

2.6. *Western blot analysis of caspase-8, -9, and -3 and PARP*

Granulosa cells were broken in homogenization buffer containing 50 mM Tris-base (pH 7.4), 150 mM NaCl, 10 mM EDTA, 0.1% Tween-20, and protease inhibitors (0.1 mM phenyl methyl-sulfonylfluoride, 5 g/mL aprotinin, and 5 g/mL leupeptin), then centrifuged at 12,000 × g for 30 min. Protein concentration was measured using a DC protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), following the manufacturer's protocol and electrophoresis was performed with the same sample amount in 10% SDS-PAGE (polyacrylamide gel electrophoresis) under reducing conditions, as previous described (*20*). The proteins separated by electrophoresis were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech), based on the procedures of Towbin *et al*. (*21*) and immersed in Tris-buffered saline (TTBS; 10 mM Tris-HCl, pH 7.6, containing 150 mM NaCl, and 0.1% Tween-20) containing 5% non-fat dry milk as a blotting method to prevent non-specific bonding. The blocked membrane was treated for 1 h in TTBS with rabbit polyclonal anti-human caspase-8, -9, and -3 and PARP antibodies at a 1:1,000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After treating 3 times for 1 min, the membranes were treated with anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) at a dilution

of 1:1,000 in TTBS for 40 min at room temperature. The signal of these membranes was visualized using a chemiluminescence solution (ECL kit; Amersham Life Science, Buckinghamshire, UK) and observed with X-ray film (Hyperfilm, Amersham Life Science).

2.7. *Statistical analysis*

Student's *t*-test was used for statistical comparisons. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. *Checking apoptosis by the TUNEL method*

DNA fragmentation was analyzed to assess apoptosis of the cultured human granulosa-luteal cells after treatment with GnRH-Ag at different doses. We confirmed that DNA fragmentation in the cultured human granulosa-luteal cells increased at a GnRH-Ag concentration > 10^{-6} M (Figure 1). On the basis of this result, GnRH-Ag at a concentration of 10^{-6} M GnRH-Ag was used in each experimental group.

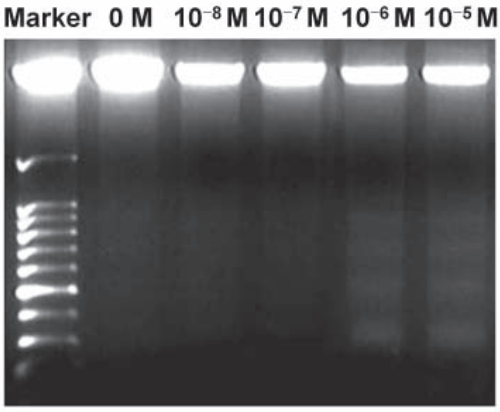

Figure 1. Analysis of DNA fragmentation in cultured human granulosa-luteal cells after GnRH-Ag treatment in a dose dependent manner. DNA fragmentation increased in concentrations over 10^{-6} M GnRH-Ag.

The TUNEL method was used to evaluate cellular death induced by apoptosis in cultured human granulosa-luteal cells. Some cellular condensation was observed using a phase contrast microscope; nuclei in these cells was condensed and fragmented. After treatment of cultured human granulosa-luteal cells with normal saline or 10^{-6} M GnRH-Ag, cellular apoptosis increased in the group of cells treated with GnRH-Ag (Figure 2). Quantitative analysis of apoptotic cells in the human granulosa-luteal cells cultured with saline or GnRH-Ag was conducted. The percent of apoptotic cells in saline compared to GnRH-Ag was 8.02 ± 5.73 *vs*. 36.83 ± 12.34. The rate of apoptosis following GnRH-Ag treatment (36.83 ± 12.34) increased significantly compared to saline treatment (8.02 ± 5.73; $p < 0.05$) (Figure 3).

3.2. *Caspase-8 -9, and -3 activity in cultured human granulosa-luteal cells*

Caspase-8, -9, and -3 activity associated with apoptosis was assessed using each fluorogenic substrate for caspase-8, -9, and -3, as described in the Methods section (*19*). Apoptotic cells displaying condensed nuclei showed intense caspase-8, -9, and -3 activity. As a result, we confirmed that the fluorogenic substrates for caspase-8, -9, and -3 activated in apoptotic cells was dissolved and green fluorescence was produced. In contrast, green fluorescence was not produced in normal cells (Figure 4).

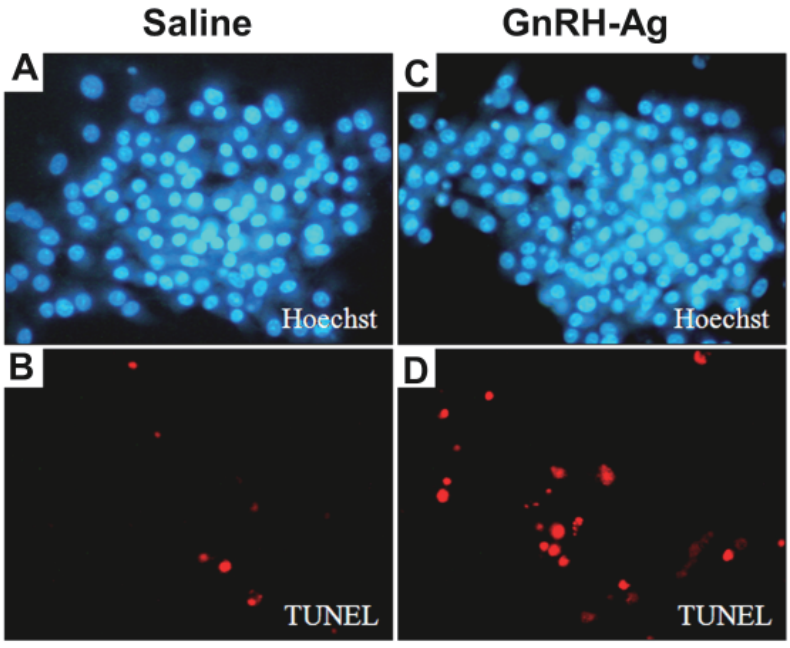

Figure 2. Evaluation of apoptosis in cultured human granulosa-luteal cells using the TUNEL method. Twenty-four hours after culture, apoptosis was detected using an *in situ* apoptosis detection kit after fixation. Nuclei were counterstained with Hoechst 33258 and observed under a fluorescence microscope **(A** and **B)**. Cells with red-colored nuclei were considered apoptotic. The number of apoptotic cells increased with GnRH-Ag **(D)** compared with saline treatment **(B)**. Original magnification, ×400.

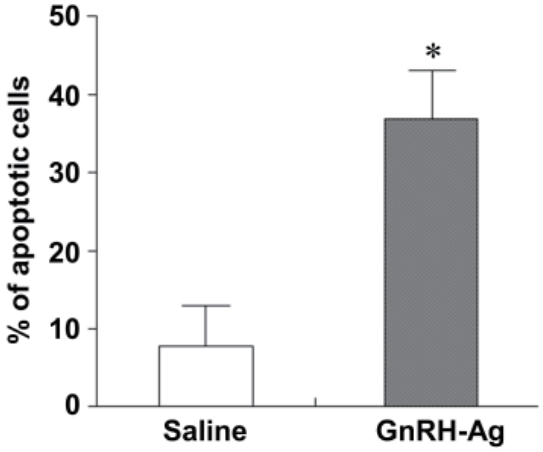

Figure 3. Quantitative analysis of apoptotic cells in human granulosa-luteal cells cultured with saline or GnRH-Ag. For the quantitative analysis of apoptosis, apoptotic cells and total cells 300 to 500 cells were counted for each slide. The rate of apoptotic cells in GnRH-Ag treatment increased significantly compared with that of saline treatment. Data points represent the mean ± S.E.M. of five independent experiments. * $p < 0.05$ compared to the corresponding saline group.

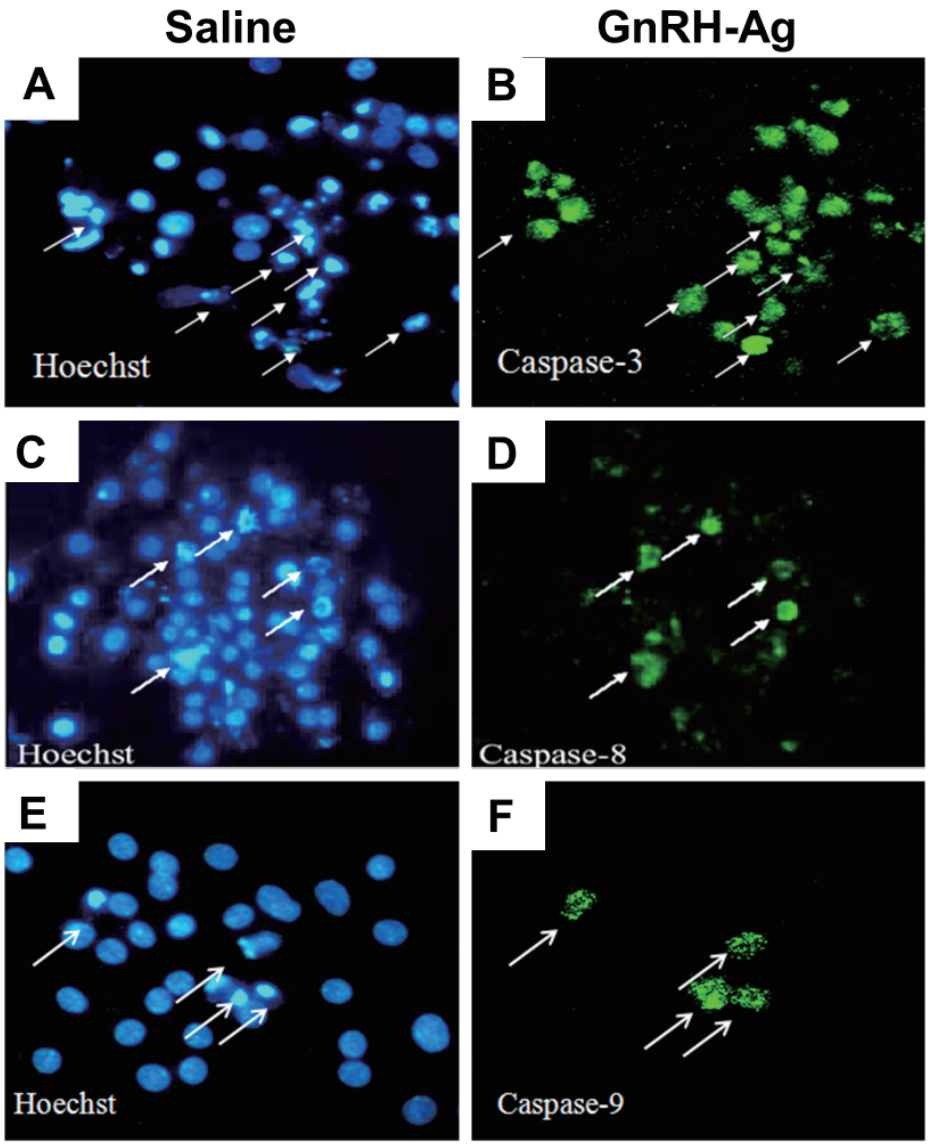

Figure 4. Caspase-8, -9, and -3 activity in cultured human granulosa-luteal cells. Caspase-8, -9, and -3 activity in the cells was assessed using a fluorogenic substrate for caspase-8, -9, and -3 as described in Methods. Apoptotic cells displaying the condensed and fragmented nucleus show the intensity of caspase-3 **(A** and **B)**, -8 **(C** and **D)**, and -9 **(E** and **F)**. Arrows indicate the cells showing activation of caspase-8, -9, and -3 stained with fluorescent substrates of caspases as a control to compare with GnRH-Ag. Original magnification, ×400.

3.3. *Increase in caspase-8, -9, and -3 and PARP activation in cultured human granulosa-luteal cells by GnRH-Ag*

Western blot analysis was used to determine whether or not apoptosis of human granulosa-luteal cells induced by GnRH-Ag was caused by activation of caspase protein. Initially, a 32 kDa inactivated pro-form of caspase-3 decreased, but the 17 kDa and 12 kDa cleaved form of caspase-3 increased following GnRH-Ag treatment (Figure 5A). A 55 kDa pro-form of caspase-8 was present in both lysates. A 40 kDa cleaved form of caspase-8 increased in the GnRH-Ag treatment (Figure 5B).

Also, a 47kDa pro-form of caspase-9 decreased, but a 37 kDa cleaved form of caspase-9 increased in the GnRH-Ag treatment group (Figure 5C). Analysis of PARP activity by Western blot, such as the final stage of apoptosis, showed that the 86 kDa cleaved form of PARP increased similar to the results of the caspases (Figure 5D).

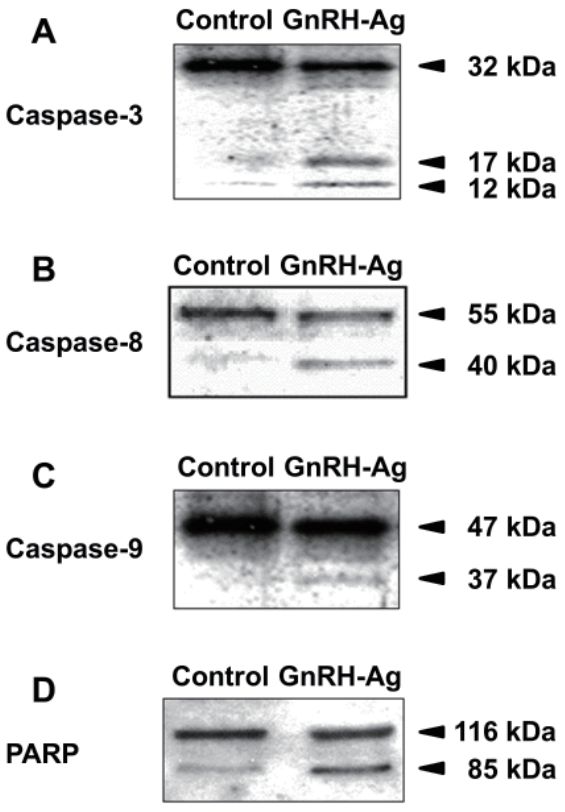

Figure 5. Western blot analysis of caspase-8, -9, and -3 and PARP in cultured human granulosa-luteal cells after GnRH-Ag treatment. Lysates from human granulosa-luteal cells treated with saline or GnRH-Ag were probed for caspase-8, -9, and -3 and PARP using anti-caspase-8, -9, and -3 and PARP antibodies. **(A)** A 32 kDa pro-form of caspase-3 was present in both lysates. A 17 kDa cleaved form of caspase-3 increased in the GnRH-Ag treatment. **(B)** A 55 kDa pro-form of caspase-8 was present in both lysates. A 40kDa cleaved form of caspase-8 increased in the GnRH-Ag treatment. **(C)** A 47 kDa pro-form of caspase-9 was present in both lysates. A 37 kDa cleaved form of caspase-9 increased in the GnRH-Ag treatment. **(D)** A 116 kDa pro-form of PARP was present in both lysates. A 86 kDa cleaved form of PARP increased in the GnRH-Ag treatment.

4. Discussion

GnRH has been shown to control the function of the ovary by direct action, as well as indirect action through the pituitary gland (*2*,*3*). Generally, the direct effect of GnRH has been shown to inhibit and promote ovarian function simultaneously; this GnRH effect on the ovary has been reported to act directly on the ovary through the GnRH receptor, which exists in ovarian granulosa-luteal cells (*22*).

It has been shown that GnRH may inhibit follicular growth and steroidogenesis in the ovary (*1*). It has also been reported that GnRH affects the cellular physiologic changes in the ovary, and oocyte growth and ovulation (*2*,*3*). This GnRH effect on ovarian function is associated with apoptosis in the ovary (*7*,*8*), but the mechanism has not yet been defined. To ascertain apoptosis in the ovary after treatment of granulosa-luteal cells with GnRH-Ag, we studied apoptosis and the associated protein changes.

After demonstrating induction of DNA fragmentation in cultured human granulosa-luteal cells by GnRH-Ag in a dose-dependent manner, DNA was extracted, and electrophoresis was performed. We confirmed DNA fragmentation increased as the concentration of GnRH-Ag in the cultured human granulosa-luteal cells increased. DNA fragmentation is a phenomenon specifically expressed in apoptotic cells and has been used as an indicator of apoptosis (*23*). DNA fragmentation in the cultured human granulosa-luteal cells increased at a GnRH-Ag concentration > 10^{-6} M (Figure 1). On the basis of this result, GnRH-Ag at a concentration of 10^{-6} M GnRH-Ag was used in each experimental group.

This dosage of 10^{-6} M GnRH-Ag that induced apoptosis is the physiological concentration. In recent research, it has been reported that GnRH-Ag is related to comparative effects on the proliferation, apoptosis, and differentiated function of cultured porcine granulosa cells from varying follicular stages (*24*). One downregulates granulosa cell proliferation in immature follicles as well as steroidogenesis in mature follicles, and the other upregulates apoptosis of granulosa cells regardless of the stage of follicular growth. Also, it has been reported that GnRH agonist increases in a dose dependent manner in the incidence of apoptotic human luteinized granulosa cells. This research suggests that clinical use of GnRH-Ag in IVF and embryo transfer (IVF-ET) should perhaps be reconsidered in the context of its apoptosis-inducing effect (*3*).

During apoptosis, nuclear changes, such as nuclear fragmentation and chromatin condensation with DNA fragmentation is known to accompany apoptosis (*25*).

Also, cellular morphologic changes following apoptosis are a phenomenon in which the cell shrinks and is split into some fragments, thus forming an apoptotic body with chromatin condensation. This apoptotic body was confirmed in our experiments, and TUNEL staining showed that cells with apoptotic bodies were stained.

The mechanism of intracellular apoptosis of the ovary by GnRH has been postulated to be induced by the pathway of protein kinase C through GnRH, thus increasing the amount of intracellular calcium and changing phosphatidylinositol (*26*,*27*). Recently, when the GnRH, buserelin, was given to white rats, it was reported to decrease the expression of inducible nitric oxide synthase (iNOS) mRNA and promote cytostasis in ovarian follicular development (*28*). Also, it has been reported that apoptosis is directly suppressed after *S*-nitroso-*N*-acetyl-D,L-penicillamine, a NO inducible product, was used to treat cultured granulosa cells (*29*). Apoptosis was suppressed after sodium nitroprusside, another NO inducible product, was used to treat rat ovary (*30*). These results suggested that an increase in apoptosis of granulosa-luteal cells by GnRH-Ag may be induced by decreasing the intracellular NO concentration, but the signal transduction mechanism has not been clearly elucidated.

Recently, the understanding of apoptosis has increased, and a variety of intracellular signal transduction materials have been implicated. It has been reported that interleukin-1b converting enzyme (ICE) family cystein protease among apoptosis signal transduction materials plays an important role in apoptosis (*31*). Fifteen additional caspases have been investigated in several organisms (*32*,*33*). Generally, caspases exist within a cell in inactive forms and become active through break-down by proteolytic enzymes. Caspase-8, -9, and -3 are among these caspases that play a central role in apoptosis.

Caspase-8 is activated in response to cellular apoptosis induction materials, such as TNF-α or Fas ligand, and becomes active in a complex associated with cytoplasmic death receptor (*34*,*35*). Caspase-9 is activated by material inducing extraction of cytochrome c from mitochondria, and requires dATP, apoptotic protease activating factor 1 (APAF-1), and connection with extracted cytochrome c for activation (*36*). Caspase-3 may expand the signals of caspase-8 and caspase-9 to result in apoptosis (*37*,*38*). We do not fully understand which role each caspase plays in the apoptosis process, but it has been shown that owing to massive research on caspase-3, that this enzyme dissolves substrates, such as PARP (*39*,*40*), inhibitor of caspase activated deoxyribonuclease (ICAD) (*41*), and gelsolin (*42*). These materials are needed to compose the intracellular skeleton, or preserve intranuclear DNA. If these materials are dissolved or activated, they induce apoptosis through cellular condensation and DNA fragmentation.

We have demonstrated that the activities of caspase-8, -9, and -3 increase within granulosa-luteal cells with nuclear condensation, a characteristic of apoptosis. We confirmed the protein of activated caspase-8, -9, and -3, by Western blot , increased during apoptosis of cultured human granulosa-luteal cells by treatment with GnRH-Ag.

As a result of our experiments, the expression of proteins of activated caspase -8, -9, and -3 increased. Similarly, activated PARP protein increased in the group of cells treated with GnRH-Ag. Taken together, we concluded that the mechanism of apoptosis of human granulosa-luteal cells involves signal transduction induced by activation of caspase-8, -9, and -3, and finally activation of PARP, thus inducing intranuclear DNA fragmentation.

Considering the function of each caspase-8, -9, and -3 as previously mentioned, we expected that sequential activation of caspase-8, -9, and -3 and cleavage of PARP may induce the apoptosis of human granulosa-luteal cells by GnRH-Ag, which warrants further investigation.

Our results are important data showing the direct induction of apoptosis in the cells of the ovary by GnRH-Ag. The apoptosis signaling transduction pathway by GnRH, delineated in part, will promote a better understanding of gynecologic endocrine disease. Through our research on GnRH-Ag used in IVF-ET procedures which directly affect apoptosis of human granulosa-luteal cells in the ovary as well as downregulation through the pituitary gland, we hope to obtain the basic data needed to develop new ovarian hyperstimulation regimens.

Moreover, our *in vitro* experimental results suggest potential mechanisms responsible for follicular atresia through regulation of apoptosis of granulosa cells by GnRH, which warrants further investigation.

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Gastrodia elata modulates amyloid precursor protein cleavage and cognitive functions in mice

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Summary ***Gastrodia elata*** **(Tianma) is a traditional Chinese medicine often used for the treatment of headache, convulsions, hypertension, and cardiovascular diseases. The vasodilatory actions of Tianma led us to investigate its specific effects on memory and learning as well as on Alzheimer's disease (AD)-related signaling. We conducted a radial arm water maze analysis and the novel object recognition test to assess the cognitive functions of Tianma-treated mice. Our data show that Tianma enhances cognitive functions in mice. Further investigations revealed that Tianma enhances the α-secretase-mediated proteolytic processing of the amyloid precursor protein (App) that precludes the amyloid-β peptide production and supports the non-amyloidogenic processing of App which is favorable in AD treatment. We hypothesize that Tianma promotes cognitive functions and neuronal survival by inhibiting β-site App-cleaving enzyme 1 activity and promoting the neuroprotective α-secretase activity.**

Keywords: Alzheimer's disease, β-Amyloid precursor protein, Kampo, Neurodegeneration, traditional Chinese medicine

1. Introduction

As the occurrence of dementia and cardiovascular disease increase with age, there has been a growing interest in developing novel protective agents because biological aging represents also the major risk factor with respect to the development of Alzheimer's disease (AD), vascular dementia (VD), and other cardiovascular diseases (CD). The number of patients suffering from AD, VD, and CD is a significant threat to the aging people all over the world. However, despite advances in technology and understanding of biological systems, drug discovery for these and other diseases is still a lengthy and expensive process. Traditional herbal medicine is especially attractive for disease prevention, health maintenance, and sicknesses that are non-responsive to current Western medicine and thus has potential benefits that attract worldwide attention and interests. The use of medicinal herbs has a long history in Asia and is commonly used to treat various neurological diseases including stroke, epilepsy and VD (*1,2*). A total of 365 plants including several orchids are listed in the earliest known Chinese Materia Medica (Shennon bencaojing (~ 100AD) or Divine Husbandman's Classic of the Materia Medica). In Bencao gangmu (Compendium of Materia Medica), the most renowned herbal text in China, three orchids that have been extensively studied and widely used as herbal medicines are *Dendrobium nobile* (Shi Hu/Shifu), *Gastrodia elata Blume* (Tianma, Orchidaceae), and *Bletilla striata*. However, current Western methodologies need to take into consideration the complex mixture of chemicals and how they are to be used in human. The scientific proof and clinical validation of these herbal formulations require a rigorous approach that includes chemical standardization, biological assays, animal models, and clinical trials (*3,4*).

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In our project, we investigated the effect of the medicinal herb Tianma on physiological and pathogenic processes related to AD by using various cellular approaches and *in vivo* mice models. Specifically, we studied the effect of Tianma on the processing of the amyloid precursor protein (App) by the α-, β-, and γ-secretases (Adam, Bace, and Psen-1/2) and cognitive functions using the radial arm water maze (RAWM) and the novel object recognition tests. Our research approach aims at gaining more insight into the potential therapeutic application of medicinal herbs, such as Tianma, to explore new potential avenues for the treatment of neurocardiovascular diseases such as AD (*5,6*).

2. Materials and Methods

2.1. *Reagents*

Unless indicated, all reagents used for biochemical methods were purchased from Sigma-Aldrich (St. Louis, MO, USA). Huperzine-A was purchased from Sigma as well.

2.2. *Animal material*

Experimental procedures, including the killing of animals, were in accordance with the International Guiding Principles for Animal Research (WHO) and were approved by the local Institutional Animal Care & Use Committee (NTU-IACUC). One-year-old mice were obtained from the laboratory animals centre (National University of Singapore). Mice (C57BL/6J) were randomly assigned to control and Tianma-treated groups (10 each, 5 male, 5 female). According to previous reports and our own recent studies, the average daily dose of Tianma per mouse was 2.5 g/kg body weight (*7-10*). Feeding was done orally with a blunt needle syringe by dispensing the Tianma solution drop by drop for the period of two months. Control mice were treated with the same volume of the solvent only. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. *Herb preparation*

Gouteng (*Uncaria sinensis* or *Ramulus cum uncis Uncariae*) was obtained from Eu Yan Sang (Singapore). The rhizome of *Gastrodia elata* (Tianma) was collected from Zhaotong City, China and provided by Dr. Jun Zhou (Kunming Institute of Botany, Chinese Academy of Science, Yunnan, China). The species was identified and chemically analyzed as reported previously (*11*). In this study, whole dried tubers of the Tianma were hammered into smaller pieces and subsequently ground to fine powder. Seven point five grams of Tianma powder was mixed with 100 mL sterilized Milli-Q water and boiled for 1 h at 100ºC. The solution was centrifuged at 5,000× g for 10 min at room temperature. The supernatant was filtered with a whatman filter paper (GE Healthcare, Chalfont St Giles, UK), yielding approximately 85 mL. The Tianma solution was concentrated at 60ºC under vacuum and the final volume was reduced to 10 mL for further applications. Gouteng was prepared accordingly. In addition, gastrodin (Kunming Pharmaceutical Corp, State New and High Technology Development Zone, Kunming, Yunnan, P.R. China) was pounded into powder, dissolved in sterile deionized water, ultra-sonicated for 2 min and filtered through a 0.45 μm Acrodisc® membrane (Pall Corporation, Singapore). For all cell-based assays, Tianma and Gouteng were applied at 1 mg/mL while gastrodin was administered at a concentration of 500 μg/mL (*12,13*).

2.4. *Cell culture*

Mouse N2a cells (kindly provided by Prof. Zhiwei Feng, SBS, NTU, Singapore) were propagated at 37°C in humidified 5% CO_2/95% air, in Dulbecco's Modified Eagle's Medium (DMEM, GlutaMax™; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen), non-essential amino acids (Invitrogen), and antibiotic-antimycotic (Invitrogen). Rat TAF-PC12 cells were cultured as described previously (*14*).

2.5. *Cell proliferation assays*

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) and the fluorescein isothiocyanate (FITC) 5-bromo-2'-deoxyuridine (BrdU) Flow Kit (BD Biosciences, Franklin Lakes, NJ, USA) were used to analyze cell proliferation as described previously (*15*). Briefly, the CellTiter-Glo® Luminescent Cell Viability Assay generates a luminescent signal based on the quantity of adenosine triphosphate (ATP) present in viable cells, which is proportional to the number of metabolically active cells. N2a cells were cultured for three passages and thereafter plated at a density of 20,000 cells/well on an opaque 96-well plate in 100 μL of complete N2a cell culture media. Tianma was added (as indicated in the text) and cells were exposed to it for 72 h. One volume of CellTiter-Glo® reagent was added and luminescence was recorded 20 min later using a plate reader luminometer (Fluoroskan Ascent FL; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Additionally, to quantify proliferating N2a cells, the FITC BrdU Flow Kit was used where 4×10^6 N2a cells were obtained and labelled with 3 mM of BrdU for 72 h. This was performed for the control cells and Tianma-treated cells. BrdU-labeled N2a cells were then stained with the FITC-conjugated anti-BrdU antibody (1:50) according

to the manufacturer's protocol and 10,000 cells were analyzed by flow cytometry (BD FACSCalibur, BD Biosciences) using the FL1 detector for BrdU-positive cells and the FL3 detector for 7-amino-actionmycin D (7-AAD)-positive cells to determine the percentage of proliferating cells (BrdU and 7-AAD-positive) among the total cell population analyzed. Results shown represent quadruplicated measurements, where each measurement consisted of three repetitions.

2.6. *Cell lysis, protein extraction, cell culture supernatant collection*

For cell lysis, adherent cells were washed in the dish using ice-cold PBS (−/−), collected using a disposable cell scraper (Greiner Bio-One GmbH, Frickenhausen, Germany) and lysed by adding a specific lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 40 mM NaF, 5 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 1% (v/v) Nonidet P-40, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 ng/mL of aprotinin and EDTA-containing protease inhibitor (Roche, Mannheim, Germany) as well as phosphatase inhibitor cocktails 1 and 2) followed by incubation on ice for 5-10 min. Lysed cells were centrifuged at 10,000 × g at 4°C for 10 min. The supernatant containing the protein extract (lysate) was either immediately used for further Western blot analyses or stored at −80°C. The cell culture supernatant of the adherent cells was used for enzyme-linked immunosorbent assay (ELISA).

2.7. *SDS-PAGE and Western blot analysis*

Twenty micrograms of cell lysates were resolved by 8-12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 0.02 A of constant current and transferred to a polyvinylidine fluoride (PVDF) membrane (0.22 µm; Amersham) using the 'semi-dry' transfer method (BioRad, Singapore) for 60 min at 0.12 A in buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, and 0.01% (w/v) SDS. The membrane was blocked with 5% BSA (BioRad) in phosphate-buffered saline (PBS) plus 0.1% Tween-20 (PBS-T) for 2 h at RT, washed three times in PBS-T for 10 min each, and incubated with primary antibody (diluted in 2% BSA in PBS-T) for overnight at 4°C. The membranes were washed as described above, incubated with HRP-conjugated secondary antibody for 1 h at RT, and developed using the ECL plus Western blot detection reagent (Amersham, Piscataway, NJ, USA). X-ray films (Konica Minolta Inc., Tokyo, Japan) were exposed to the membranes before film development in a Kodak X-OMAT 2000 processor (Kodak, Ontario, Canada). For equal sample loading, protein quantification was performed using a '2D Quant' kit (Amersham) with at least two independent replicates. BSA was used as a standard for protein quantification. To re-probe the same membrane with another primary antibody, Pierce's (Pierce Biotechnology, Inc., Rockford, IL, USA) 'stripping solution' was used to strip the membranes. In addition, equal sample loading was confirmed using Gapdh (Glyceraldehyde 3-phosphate dehydrogenase) as a reference protein. Western blot experiments were performed at least four times for statistical quantification and analyses (n = 4), and representative blots are shown. Values (= relative protein expression) represent the ratio of densitometric scores (GS-800 Calibrated Densitometer and Quantity One quantification analysis software version 4.5.2; BioRad) for the respective Western-blot products (mean ± S.D. (standard deviation)) using the Gapdh bands as a reference.

2.8. *Quantification of soluble App-alpha (sApp-α) by ELISA*

The various cell culture supernatants collected were centrifuged at 5,000 × g for 10 min. Concentrations of sApp-α in the supernatants were measured by solid phase sandwich ELISA using the mouse/rat sApp-α-specific assay kit (#27415; ARP American Research Products Inc., Belmont, MA, USA). To measure the concentration of human soluble Tumor necrosis factor receptor superfamily member 1B (hsTNFRSF1B), cleaved specifically extracellularly by α-secretase and resembling sApp-α from TAF-PC12 cells (*14*), the ELISA technique was employed and analyzed according to the manufacturer's protocol (Quantikine® DRT200; R&D Systems, Minneapolis, MN, USA). Absorbance measurements were taken at 450 nm using a Safire2™ microplate reader (Tecan Group Ltd., Männedorf, Switzerland) and analysis was done using the Magellan™ V5.01 (Tecan) software. Experiments were performed four times in triplicates. Data are presented as sApp-α levels in pg/mL compared to controls. Statistical evaluation of results was performed using analysis of variance (ANOVA) and statistical error was indicated as mean ± S.D.

2.9. *Antibodies*

Anti-β-site App-cleaving enzyme (Bace1) (1:1,000, mouse monoclonal; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (1:5,000, mouse monoclonal; Santa Cruz) and anti-App (1:10,000, rabbit polyclonal; Sigma-Aldrich).

2.10. *RAWM test to analyze cognitive functions in Tianma-treated mice*

Behavioral testing of mouse models is essential for establishing the progression of mnemonic deficits as

mice age. One of the most elegant, efficient and simple analysis is the Morris water maze (MWM) which is a simple spatial task based on visual cues in which a rodent swims in a tank to find a hidden platform. There are an increasing number of memory tasks including the MWM, the Y-maze, fear conditioning, conditioned food avoidance and object recognition testing. However, the recently found RAWM seems to be the most reliable task for detecting memory deficits in mice models and to study more efficiently learning performance while the test robustly discriminates between mice that learn well and those that learn poorly (*16*).

The RAWM is a hybrid of the MWM and a radial arm maze, which takes advantage of the simple motivation provided by immersion into water, together with the benefits of scoring errors (rather than time or proximity to platform location) associated with the radial arm maze. The RAWM contains six swim paths (arms) extending out of an open central area, with an escape platform located at the end of one arm (the goal arm). The goal arm location remains constant for a given mouse. The RAWM has the spatial complexity and performance measurement simplicity of the dry radial arm maze combined with the rapid learning and strong motivation observed in the MWM without requiring foot shock or food deprivation as motivating factors. Our mice were treated for two months with Tianma (~ 2.5 g/kg/day) before the RAWM test was performed. For RAWM, on day 1, mice were trained for 20 trials (spaced over 3 h), with trials alternating between visible and hidden platform. On day 2, mice were recorded for 20 trials with the hidden platform. Entry into an incorrect arm was scored as an error. If a mouse did not leave its start arm within 15 sec then it was also counted as an error. Each indicated block of trials consists of 4 trials. The data shown were obtained from Tianma-treated mice (squares, n = 10) and untreated littermates (circles, n = 10). The data shown were collected independently on two separate days with new mice each time. Statistical analysis was based on univariate and multivariate one-factor ANOVA, and between-group comparisons were made by Tukey's test.

2.11. *Novel object recognition test on Tianma-treated mice*

Experiments were performed as described previously (*17-19*). In the one-trial object recognition task, animals were exposed to two different objects which they have to identify as novel or familiar based on the memory of an earlier experience with one of the two objects they encountered in the same open-field. Memory involved in one-trial object recognition is that of an episodic type in the life of these animals. The one-trial object recognition task is limited to memory of an object, the location of an object and the context in which an object was encountered. It only provides measure of memory of when such encounter with an object, a place and/or a context took place (episodic memory), if a significant long temporal delayed dimension of an episode (*e.g.* 24 h as in our study) has been incorporated. If an object is completely novel, it will always attract attention and induce more exploration even when presented alongside a preferred object. Attention and motivation processes are likely to prioritize novelty detection. Anything new is alerting and needs to be examined. However, such level of exploration induced by object novelty can be observed within a short time window after which it would decline in favor of more exploration of a familiar preferred object. The apparent unconditioned preference for a novel object is considered as an indication that a representation of the familiar object exists in memory. Briefly, in our study all mice were given a habituation session which allowed each of them to explore the empty apparatus for 5 min. On the training session, each mouse was placed in the middle of the box with two identical objects (A1 and A2) located at both ends. Each mouse was allowed to explore for 3 min. The mouse was then removed and returned to its home cage. After one hour, the test began. Each mouse was placed into the box where an identical object and a new object (A1 and B) were introduced at two sides of the box. The mouse could explore for 3 min. The time spent to explore the novel object and the time spent to explore both objects and the box was measured. This is to test the short-term memory. After 24 h, the mouse was placed into the box again, with the identical object A1 and another novel object C. The time spent to explore the novel object and the total exploring time was measured as the day before. The parameters analyzed were percentage of time that the animals explored identical objects during the training and the percentage of time that the animals explored the novel object at 1 h (working memory or short term memory) and 24 h (episodic memory or long term memory) after training. We considered this last parameter also as an index of memory retention. Results are expressed as percentage of novel object recognition time.

2.12. *Statistical evaluation*

The data obtained in this investigation are illustrated as mean ± S.D. Differences between the groups were established using an unpaired Student's *t*-test while within-group comparisons were performed using the paired Student's *t*-test. SPSS (Statistical Products and Service Solutions) for Windows Version 19 was used to perform ANOVA followed by Fisher's Protected Least Significant Difference (PLSD) *post hoc* tests, when warranted. To be considered statistically significant,

we required a probability value to be at least < 0.05 (95% confidence limit, * $p < 0.05$).

3. Results

In the present study, we sought to find a lead in the potential use of Tianma as a therapeutic agent for the prevention of AD. Using neurocellular *in vitro* experiments and *in vivo* animal models, the effect of Tianma on neural signaling cascades and on cognitive functions have been investigated.

3.1. *Effect of Tianma on cognitive function in mice using RAWM test*

To determine the effect of Tianma on cognitive functions, we applied the recently established RAWM test to thoroughly discriminate between mice that learn well and those that learn poorly in a complex memory and learning task (*16*). Interestingly, after the treatment of the mice over a period of two months, Tianma produced a significant improvement of the learning task (Figure 1).

3.2. *Effect of Tianma on novel object recognition test*

To further validate our observation, we performed the novel object recognition test. In this test, when exposed to a familiar object alongside a novel object, young and adult mice usually approach frequently and spend more time exploring the novel than the familiar object. However, there are instances where preference for novelty is supplanted by preference for familiarity. For instance, there is a growing experimental evidence showing preference for familiarity in young and old infants (*20,21*). A reduced preference for a novel object can be accounted for by the physical properties of the novel object which may not require too much attention or by some affective attributes invested into the familiar object. For these kinds of reasons, a possibility has always been envisaged where a familiar object can be preferred to a novel object (*22*). Besides, the level of exploration induced by object novelty can be observed within a very short time window after which it would decline in favor of more exploration of a familiar preferred object. Novelty-preference is short-lived and lasts for the time necessary for mice to encode the physical properties of the stimulus induced by the novel object. Thus, it seems that Tianma-treated mice learned faster and were more quickly able to explore the new object than control mice and afterwards spent longer time with the familiar objects (Figure 2) – therefore, confirming the learning-task results obtained from the RAWM experiments (*19*).

3.3. *Effect of Tianma on App cleavage in neuronal N2a cells*

To determine a potential link between the improved cognitive functions caused by the application of Tianma and prevention of dementia such as AD, we studied a possible molecular mechanism responsible for this finding. Since App is the pivotal protein involved in the molecular cascade ultimately leading to AD (*23,24*), we characterized the effect of Tianma on the expression of the App protein. However, no changes in full length App expression could be detected though a slight increase in the C-terminal fragments (CTFs) could be observed (Figure 3A),

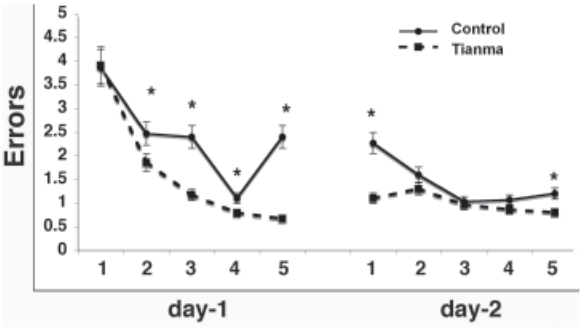

Figure 1. Tianma improves cognitive functions in mice. RAWM testing of Tianma-treated mice. Average error committed by one-year-old Tianma-treated (for 2 months) mice (squares, $n = 10$) and control littermates (circles, $n = 10$). Experiments were performed as described in Materials and Methods. Each block (1-5) consists of four trials. The data shown were obtained from Tianma-treated mice (squares, $n = 10$) and untreated littermates (circles, $n = 10$). The data shown were collected independently on two separate days with new mice each time. Data are presented as the mean ± S.D. (by ANOVA) (* $p < 0.05$ compared with controls).

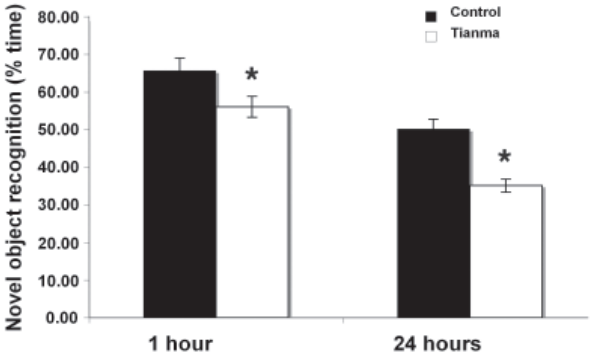

Figure 2. Effect of Tianma on memory performance in an object recognition test. Experimental procedure was carried out according to Materials and Methods. Mice were familiarized with various objects for a specific time period before again confronted with known and new objects after a time interval of 1 h and 24 h, respectively. Compared with the control group, Tianma-treated mice showed remarkable differences for the time spent to explore the novel and the familiar object, suggesting that the Tianma-treated mice have a better learning performance in recognizing novel objects faster and remembering the familiar object at both after 1 h and 24 h, respectively. Data presented are mean (percentage time = $t_{novel}/(t_{novel} + t_{familiar}) \times 100$) ± S.D. (* $p < 0.05$).

probably initiated through an enhanced α-secretase-mediated App-cleavage. Therefore, we studied the proteolytic cleavage of App in neuronal N2a cells and the results obtained revealed that Tianma increased the sApp-α release in our *in vitro* cell culture system while the control herb Gouteng did not induce any significant changes (Figure 3B). To test whether this increased sApp-α cleavage was eventually due to an enhanced App protein expression or an increase in cell proliferation, we investigated both of it and found that neither App expression nor N2a proliferation was affected by Tianma (Figures 3C and 3D).

3.4. *Effect of Tianma on α-secretase-mediated App cleavage in TAF-PC12 cells*

To further elaborate on this interesting observation, we shifted to our previously established novel cellular system that is suitable to study App signaling as a classical ligand-receptor system and is particularly useful for the study of α-secretase-mediated App cleavage (*14*). We used TAF-PC12 cells to study specifically the effect of Tianma on App processing by the α-secretase. We found that also in this system Tianma enhanced sApp-α production, thus indicating

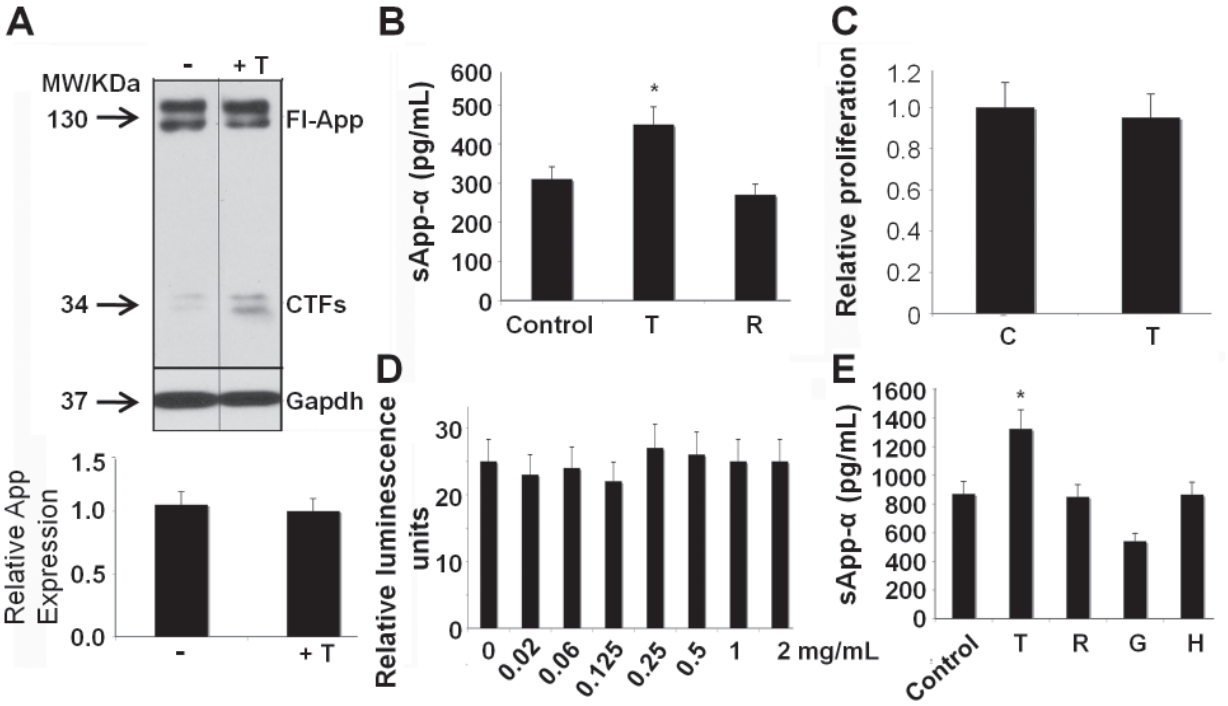

Figure 3. Effect of Tianma and Gouteng on App expression, soluble App release and cell proliferation. (A) Western blot analysis (A, top) of Tianma-treated N2a cells reveals no changes in App protein expression levels. N2a cells were grown and treated with Tianma (1 mg/mL) for 24 h as described in "Materials and Methods". Gapdh was used as the reference protein. Fl-App = full length App, CTFs = C-terminal fragments of full-length App. Western blot experiments were performed at least four times for statistical quantification and analyses ($n = 4$). Values (= relative protein expression) represent the ratio of densitometric scores for the respective Western blot products (mean ± S.D.) using the Gapdh bands as reference. No significant difference in full length App expression levels could be observed between control and Tianma-treated N2a cells (A, bottom). The slight increase in the C-terminal fragments (CTFs) was probably initiated through an enhanced α-secretase-mediated App-cleavage (A, top). **(B)** Estimation of sApp-α levels in the cell culture supernatant of neuronal N2a cells treated with Tianma and measured by ELISA. N2a cells were grown and treated with Tianma (1 mg/mL) or Gouteng (R) (1 mg/mL) for 24 h as described in Materials and Methods. Experiments were performed four times in triplicates. Data are presented as sApp-α levels in pg/mL compared to controls. Statistical evaluation of results was performed using ANOVA and statistical error was indicated as mean ± S.D. (* $p < 0.05$). Only Tianma-treated cells showed a significant elevation of sApp-α levels. **(C)** The effect of Tianma on BrdU-positive cell proliferation. N2a cells were cultured for three passages before BrdU analysis of proliferation was performed in the presence of Tianma. Quantification of BrdU incorporation is represented as the mean (± S.D.) of the percentage of BrdU-positive cells in the experimental population obtained from quadruplicated measurements, where each measurement consisted of three repetitions. No significant difference could be observed between control and Tianma-treated cells. **(D)** Dose-dependent proliferation analysis of N2a cells treated with Tianma. In the proliferation assay based on ATP-consumption, control N2a cells were cultured in N2a cell culture media as mentioned in "Materials and Methods", while experimental samples were exposed to Tianma for 72 h at various concentrations as indicated. Values represent mean luminescent units (± S.D.) obtained from quadruplicated measurements, where each measurement consisted of three repetitions. No significant difference could be observed between control and Tianma-treated cells. **(E)** Estimation of sApp-α levels in the cell culture supernatant of neuronal TAF-PC12 cells treated with Tianma and measured by ELISA. TAF-PC12 cells were grown and treated with Tianma (T) (1 mg/mL), Gouteng (R) (1 mg/mL), gastrodin (G, 500 μg/mL), or hyperzine-A (H, 10 μM) for 24 h as described in Materials and Methods. Experiments were performed four times in triplicates. Data are presented as sApp-α levels in pg/mL compared to controls. Statistical evaluation of results was performed using ANOVA and statistical error was indicated as mean ± S.D. (* $p < 0.05$). Only Tianma-treated cells showed a significant increase of sApp-α levels. Gastrodin alone had rather an inhibitory effect.

enhanced cleavage of App by α-secretase. As control, the other herb Gouteng, gastrodin (one active component of Tianma) and huperzine-A (a bioactive compound of *huperzia serrata* known to act as an acetylcholinesterase inhibitor and also described to affect App proteolytic processing (*25-28*), were included in this study, but had no significant effect on sApp-α production (Figure 3E).

3.5. *The potential mechanism of Tianma-mediated App cleavage in neuronal N2a cells*

To investigate the effect of Tianma on App receptor signaling, N2a cells were subjected to Tianma treatment for a period of 24 h. While sApp-α levels were increased as described in Figure 3, we examined the potential mechanism involved in this process by checking the expression levels of various secretases (α-, β-, and γ- such as Adam10, Adam17, Bace1/2, and Psen1/2) known to be involved in the processing of App (*23*). Accordingly, the expression of the β-secretase (Bace1) was inhibited by Tianma (Figure 4). The expression levels of the other secretases were unchanged (data not shown) – though their activity still could be affected.

4. Discussion

Herbs with potential neuroregenerative activities that have been identified include *Curcuma longa, Zingiber officinale, Huperzia serrata, Nigella sativa, Rhizoma acori graminei, Verbena officinalis* Linn, and Tianma (*4,13,28-31*). According to ancient Chinese medical literature, Tianma is an herbal medicine for the control of the internal movement of wind. The dry tuber of Tianma has long been officially listed in the Chinese Pharmacopoeia and is used in treating headaches, dizziness, tetanus, epilepsy, infantile convulsions, numbness of the limbs and also for the improvement of cognitive functions (*4,31-36*). From scientific investigations, Tianma possesses anti-oxidative and free radical scavenging (*37*), neuroprotective (*38*), and anti-depressant effects (*7,39,40*). Tianma, *huperzia serrata* and other herbs have recently also been discussed as a relevant source of potential therapeutic remedies that target AD symptoms or the primary pathogenic processes of AD especially as researchers are now exploring the possibility of a connection between AD, VD, diabetes mellitus (type 2, DMT2), and CD (*5,41,42*). The risk for dementia is particularly high when diabetes mellitus occurs together with severe systolic hypertension or heart diseases (*43,44*). The strong association of cardiovascular risk factors with AD and VD suggests that these diseases share some biological pathways in common and were discussed recently (*5,45*). Epidemiological and clinico-pathological data have indicated that antihypertensive drugs show protective effects in reducing the risk of dementia and data suggest overlaps between AD and cerebrovascular lesions that may magnify the effect of mild AD pathology and promote the progression of cognitive decline or may even precede neuronal damage and dementia (*46*). Therefore, the contribution of CD to AD and VD foreshadows that cardiovascular therapies might prove useful in treating or preventing AD and dementia (*47-52*). In traditional herbal medicine practice, synergistic and or antagonistic therapeutic efficacy among the herbs in any one prescription plays an important role in the treatment of illness. These observations may explain the medicinal effects related to AD and VD observed for Tianma and some other herbs (*28-31,33-35,53-62*). For instance, Gouteng has been traditionally used alongside with Tianma in treating high blood pressure (*63*). A recent study has illustrated that the use of Tianma and Gouteng with two other herbs has anti-hypertensive effects and shows an enhancing effect on cognitive functions and thus, taken our results into account, could be used against cognitive defects and dementia (*8,9*). However, the complexity and multi-actions of herbal bioactive compounds remain unclear. A variety of studies were carried out to purify the herbs' effective constituents and to investigate their biological functions. Data revealed that huperzine-A shows neuroprotective function and can ameliorate learning dysfunction in AD patients (*27,28,53,54,62*). Gastrodin and vanillin, derived from Tianma, were shown to exert sedative and anticonvulsive effects. Gastrodin seems to be a

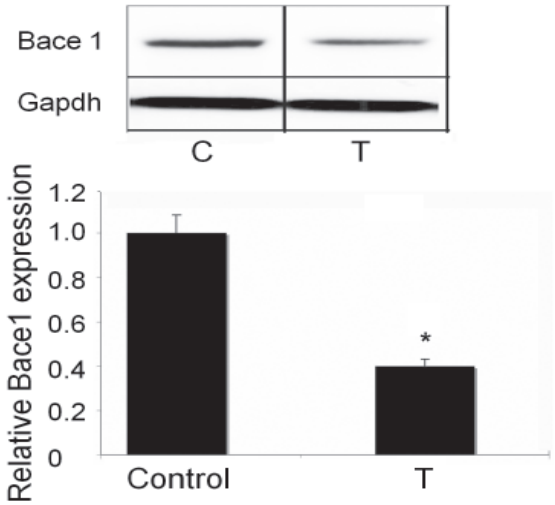

Figure 4. Relative Bace1 expression in N2a cells treated with Tianma and evaluated by Western blot analyses. N2a cells were grown and treated with Tianma (1 mg/mL) for 24 h as described in Materials and Methods. Top: representative Western blot results for Bace1 and Gapdh; bottom: statistical evaluation of the Western blots. Gapdh was used as the reference protein. Western blot experiments were performed at least four times for statistical quantification and analyses (n = 4). Values (= relative protein expression) represent the ratio of densitometric scores for the respective Western blot products (mean ± S.D.) using the Gapdh bands as a reference. Tianma-treated cells clearly show a significant reduction of Bace1 expression (* $p < 0.05$).

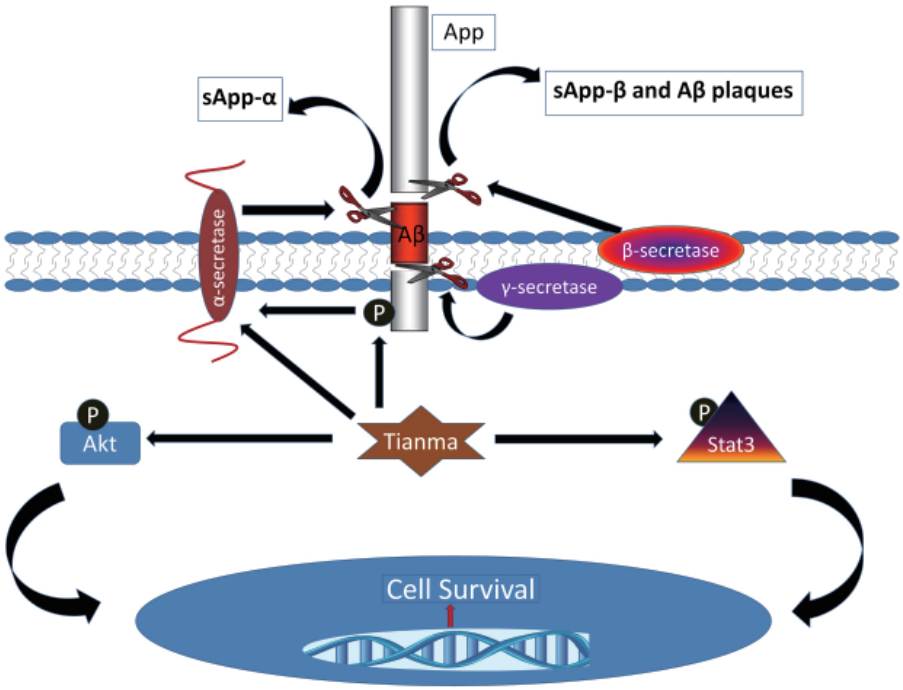

Figure 5. Schematic illustration of the action of Tianma. Tianma may become a potential therapeutic agent for the treatment of AD. Tianma's abilities in enhancing the α-secretase' activity and possible phosphorylation of App greatly encourage the proteolytic processing of App towards the non-amyloidogenic pathway that results in increased production of the neuroprotective sApp-α (*68*). Tianma's inhibitory effect on the β-secretase precludes amyloid-β peptide generation and prevents AD. It may also enhance the phosphorylation of Akt and Stat3 that aid in cell survival and retards AD pathogenesis.

safe and effective drug for treating anxiety, insomnia, neurasthenia, and mental hyper-excitation while vanillin is presumably an anti-convulsive agent (*4,31*). In addition to gastrodin and vanillin, other bioactive components of Tianma include vanillyl alcohol, 4-hydroxybenzylaldehyde, and 4-hydroxybenzyl alcohol (*64*) with gastrodin being the primary active ingredient (*65*). The importance of other secondary metabolites such as flavones and anthocyanins has been largely neglected. According to the research, gastrodin exhibits also an anti-coagulant effect (*66*) and protects cerebral cortical and hippocampal cells against amyloid-β peptide-induced neurotoxicity (*67*) which suggests that gastrodin could play a pivotal role in AD treatment. Though, in our study, another component besides gastrodin seems to be responsible for the specific effect on enhanced sApp-α cleavage (Figure 5).

As herbs contain multiple compounds with potentially versatile modes of action, the modernization and the acceptance of traditional herbal medicine into mainstream medicine in the past is restricted by the problems of unauthenticated raw material, unknown mechanisms of action, unknown bioactive compounds, non-standardization of herbal products with respect to active ingredients and lack of toxicology and safety data. However, the recent introduction of good manufacturing practice has resulted in herbs with standardized bioactive compounds. The pharmacodynamics, pharmacokinetics, safety, and efficacy of bioactive compounds can be examined in animal models according to the US-FDA' published guidance for industry on the policy for botanical drug product development which enables the use of fingerprint to show lot-to-lot consistency so that there is no need to identify the function of each individual component. If a botanical drug has been used historically to treat a disease, then the combination of Phase-I and -II for a drug development is allowed. Thus, our current study used a standardized Tianma (*11*) and provides an additional interesting insight into the molecular and cellular mechanisms of herbal medicine that may guide us to the further identification of the bioactive components (by phytochemistry) that may contribute to Tianma's potential cognitive function-enhancing activities. However, a more systemic biology study is necessary to understand the neurovascular and neuroprotective functions of herbs such as Tianma and to unravel the medically active components in Tianma (*4,69,70*).

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BST
BioScience Trends

Guide for Authors

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2. Submission Types

Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

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Case Reports should be detailed reports of the symptoms, signs, diagnosis, treatment, and follow-up of an individual patient. Case reports may contain a demographic profile of the patient but usually describe an unusual or novel occurrence. Unreported or unusual side effects or adverse interactions involving medications will also be considered. Case Reports should not exceed 3,000 words in length (excluding references).

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