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Antenatal taurine reduces cerebral cell apoptosis in fetal rats with intrauterine growth restriction

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Research Highlights

(1) Increased brain cell apoptosis in intrauterine growth-restricted fetal rats is a key reason for unfavorable long-term prognosis of the nervous system.
(2) Taurine supplement in pregnant rats noticeably reduced cell apoptosis, promoted glial cell line-derived neurotrophic factor expression and decreased caspase-3 expression in brain tissue of intrauterine growth-restricted fetal rats.
(3) Experimental results provided theoretical evidence for preventing or lessening brain injury and promoting brain development in intrauterine growth-restricted fetuses using a suitable measurement before parturition.

Abstract

From pregnancy to parturition, Sprague-Dawley rats were daily administered a low protein diet to establish a model of intrauterine growth restriction. From the 12th day of pregnancy, 300 mg/kg rine was daily added to food until spontaneous delivery occurred. Brain tissues from normal neonatal rats at 6 hours after delivery, neonatal rats with intrauterine growth restriction, and neonatal rats with intrauterine growth restriction undergoing taurine supplement were obtained for further experiments. The terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling assay revealed that the number of apoptotic cells in the brain tissue of neonatal rats with intrauterine growth restriction significantly increased. Taurine supplement in pregnant rats reduced cell apoptosis in brain tissue from neonatal rats with intrauterine growth restriction. nohistochemical staining revealed that taurine supplement increased glial cell line-derived neurotrophic factor expression and decreased caspase-3 expression in the cerebral cortex of intrauterine growth-restricted fetal rats. These results indicate that taurine supplement reduces cell apoptosis through the glial cell line-derived neurotrophic factor-caspase-3 signaling pathway, resulting in a protective effect on the intrauterine growth-restricted fetal rat brain.

Key Words

neural regeneration; intrauterine growth restriction; fetal rats; brain; neural cells; taurine; cell tosis; glial cell line-derived neurotrophic factor; caspase-3; neural development; grants-supported paper; neuroregeneration

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Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Care and Use Committee of General Hospital of Beijing Military Command, China.

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INTRODUCTION

Intrauterine growth restriction refers to poor growth of a baby while in the mother's womb during pregnancy, and is said to occur in term infants (> 37 weeks' gestation) who weigh < 2 500 g[1]. The incidence of intrauterine growth restriction is approximately 6.4%, and perinatal mortality is 10 times that of appropriate for gestational age infants[1].

Therefore, there are abundant basic and clinical studies addressing intrauterine growth restriction, especially in the area of high risk factors, onset mechanism, and hazards to the fetus. Intrauterine growth restricted children have a lower body mass, shorter body length and shorter head circumference when compared with normal newborns[1-8]. Moreover, there are an increased number of perinatal complications such as fetal distress, birth asphyxia, meconium aspiration, hypoglycemia, dysmetabolism, erythrocytosis, injury to the lungs and hepatic injury, and long-term complications such as coronary arteriosclerotic heart disease, hypertension, type II diabetes mellitus, kidney disease and neonatal chronic pulmonary disease[1-8].

Many studies have focused on the effects of intrauterine growth restriction on fetal brain development[9-10]. During the fetal period, especially in the 10^{th}–18^{th} weeks of gestation, neurons are rapidly proliferating. If the fetus encounters adverse factors, neuronal proliferation and axonal growth are directly affected[11], which would severely affect the number and volume of brain cells and cell junctions[11]. Intrauterine growth restriction not only increases perinatal morbidity and mortality rate, but also leads to stunted development, movement and behavior disorders, decreased learning ability and transient attention[12]. Intrauterine growth restriction is a key factor for the occurrence of cerebral palsy[13]. The harmful effects of intrauterine growth restriction on fetal brain development originate in the womb. Therefore, it is difficult to obtain ideal outcomes through postnatal intervention. Taken together, active prenatal intervention is of great importance to the optimal prognosis of the intrauterine growth restricted fetus.

Taurine is the most abundant amino acid in the central nervous system and a conditionally essential amino acid for growth and development. In addition, taurine can regulate osmotic pressure, stabilize the cell membrane, maintain calcium homeostasis, and improve learning and memory abilities[14]. Recently, the neuroprotective effect of taurine has received attention. A previous study showed that taurine partially or totally antagonized glycine receptors and γ-aminobutyric acid receptors, increased chloride transduction in the cell membrane, induced hyperpolarization, and inhibited neurotoxicity to excitatory amino acids[15].

Furthermore, taurine regulated calcium channels, maintained intracellular and extracellular calcium homeostasis, prevented calcium overload[16], removed *in vivo* redundant free radicals, enhanced antioxidase activity, reduced the production of lipid peroxidation products, stabilized biomembranes, exerted an obvious antioxidant effect[16], diminished caspase-8 and caspase-9 expression, and suppressed ischemia/hypoxia-induced brain cell apoptosis[17-18]. Our previous pilot study indicated that taurine supplementation in pregnant rats promotes brain cell proliferation, reduces brain cell apoptosis, and improves brain ultrastructure in fetal rats with intrauterine growth restriction[19]. However, the precise mechanisms remain unclear. Glial cell line-derived neurotrophic factor is a member of the transforming growth factor-β superfamily and exerts a neurotrophic effect[20-21]. Caspase-3 is an apoptosis-executing factor[22].

In this study, we investigated the effects of taurine supplementation on glial cell line-derived neurotrophic factor and caspase-3 expression in the brain tissues of intrauterine growth restricted fetal rats and explored whether taurine supplementation exerted a protective effect through the glial cell line-derived neurotrophic factor-caspase-3 pathway.

RESULTS

Quantitative analysis of animals

A total of 15 pregnant rats were equally and randomly assigned to three groups (n = 5 per group): control group (normal raising), model group (intrauterine growth restriction model was established using a low protein diet), and taurine group (intrauterine growth restriction model + taurine supplement). Fetal rats at 6 hours after birth from each group were used for experiments.

Effects of taurine supplementation in pregnant rats on weight of neonatal rats

The control group contained 65 fetal rats, with an average weight of 6.36 ± 0.44 g. The model group contained 60 fetal rats, with an average weight of 4.55 ± 0.45 g. The incidence of intrauterine growth restriction was 100%. The taurine group contained 59 fetal rats, with an average weight of 5.11 ± 0.67 g, with the incidence of intrauterine growth restriction being 76.27%. Compared with the control group, the weight of fetal rats was significantly lower in the model group (t = 14.104; P < 0.01). The weight of fetal rats in the taurine group was significantly higher than that in the model group (t = 7.922, P < 0.01).

Taurine supplementation in pregnant rats reduced cell apoptosis in the fetal rat brain

Taurine supplementation resulted in a decrease in terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling (TUNEL)-positive cells, chromatin condensation and brown pyknosis. Under a light microscope, apoptotic cells were found in the brain tissue of control group fetal rats. The number of TUNEL-positive cells increased in the brain tissue of model group fetal rats. There were significant differences in TUNEL-positive cells between the model group and control group (P < 0.01). The number of TUNEL-positive cells was significantly lower in the taurine group when compared with the model group (P < 0.01), but the number of TUNEL-positive cells was greater in the taurine group when compared with the control group (P < 0.01; Figure 1, Table 1).

Taurine supplementation in pregnant rats promoted glial cell line-derived neurotrophic factor protein expression in the cerebral cortex of fetal rats

Immunohistochemical staining revealed that glial cell line-derived neurotrophic factor expression was mainly found in the cytoplasm. In the control group, only a few glial cell line-derived neurotrophic factor-positive cells were visible in the cerebral cortex of fetal rats. The number of glial cell line-derived neurotrophic factor- positive cells in the cerebral cortex of fetal rats was significantly greater in the model group than in the control group (P < 0.01). The number of glial cell line-derived neurotrophic factor-positive cells was significantly higher in the taurine group than in the model group (P < 0.01; Figure 2, Table 1).

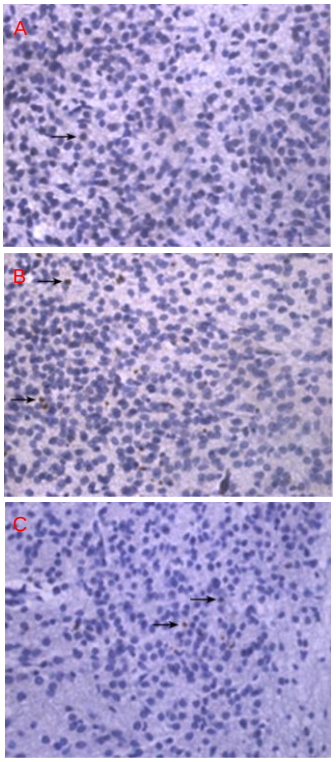

Figure 1 Effects of taurine supplementation in pregnant rats on cell apoptosis in the cerebral cortex of fetal rats with intrauterine growth restriction (terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling staining, × 400).

Apoptotic cell: There is a decrease in cell volume, chromatin condensation and pyknosis (brown; arrows).

(A) A few apoptotic cells in the control group.

(B) A large number of apoptotic cells in the model group.

(C) Cell apoptosis is reduced in fetal rats after taurine supplementation in pregnant rats.

Taurine supplementation in pregnant rats inhibited caspase-3 expression in the cerebral cortex of fetal rats

Immunohistochemical staining revealed that caspase-3 expression was mainly found in the nuclei. In the control group, only a few caspase-3-positive cells were observed in the cerebral cortex of fetal rats, showing weak staining. The number of caspase-3-positive cells significantly increased in the model group (P < 0.01). The

number of caspase-3-positive cells was less in the taurine group when compared with the model group ($P < 0.01$), but was greater than that in the control group ($P < 0.01$; Figure 3, Table 1).

Table 1 Effects of taurine supplementation in pregnant rats on the number of terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling (TUNEL)-, glial cell line-derived neurotrophic factor (GDNF)- and caspase-3-positive cells in the cerebral cortex of fetal rats with intrauterine growth restriction (cells/400-fold visual field)

Group	TUNEL-positive cells	GDNF-positive cells	Caspase-3-positive cells
Control	0.46±0.11	93.56±6.73	7.50±2.31
Model	14.76±3.42[a]	120.36±6.23[a]	151.32±24.43[a]
Taurine	6.78±1.93[ab]	139.56±5.28[ab]	37.28±11.27[ab]
Statistical value	*H*=429.80	*F*=715.17	*H*=132.543
P	0.000	0.000	0.000

The results are expressed as mean ± SD of ten rats in each group. Five non-overlapped fields of each section were selected. [a]$P < 0.01$, *vs*. control group; [b]$P < 0.01$, *vs*. model group. TUNEL- and caspase-3-positive cell counting results were analyzed using the Levene's test, and heterogeneity of variance. Intergroup comparisons were performed using the Kruskal-Wallis rank sum test. Paired comparison was performed using the Tamhane's test. GDNF-positive cell counting results were analyzed using Levene's test and homogeneity of variance. Intergroup comparison was performed using one-way analysis of variance followed by Student-Newman-Keuls test.

DISCUSSION

Results demonstrated that the mean weight of fetal rats with intrauterine growth restriction was 4.55 ± 0.45 g, and the mean weight of normal fetal rats was 6.36 ± 0.44 g, which was significantly different and indicated successful establishment of an intrauterine growth restriction model. Experimental results suggested that (1) glial cell line-derived neurotrophic factor expression was obviously more in brain tissues from fetal rats with intrauterine growth restriction than in normal fetal rats. Taurine supplementation in pregnant rats further promoted glial cell line-derived neurotrophic factor expression in brain tissues of fetal rats with intrauterine growth restriction. (2) Caspase-3 expression significantly increased in brain tissues of fetal rats with intrauterine growth restriction, and taurine supplementation in pregnant rats obviously decreased caspase-3 expression in brain tissues of fetal rats with intrauterine growth restriction. (3) Cell apoptosis remarkably increased in fetal rats with intrauterine growth restriction, but the number of apoptotic cells was dramatically reduced after taurine supplementation in pregnant rats.

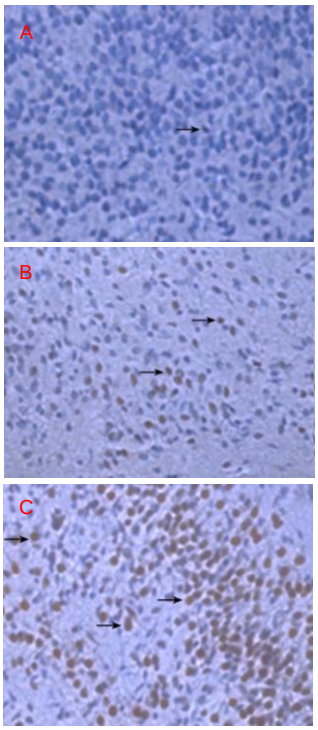

Figure 2 Effects of taurine supplementation in pregnant rats on glial cell line-derived neurotrophic factor (GDNF) expression in the cerebral cortex of fetal rats with intrauterine growth restriction (immunohistochemical staining, × 400).

GDNF expression is mainly found in the cytoplasm (brown; arrows).

(A) A few GDNF-positive cells in brain tissue of fetal rats in the control group.

(B) The number of GDNF-positive cells (darkly stained) is higher in the model group than that in the control group.

(C) The number of GDNF-positive cells (stained brown) is higher in the taurine group than that in the model group.

Apoptosis plays a crucial role in developing and maintaining the health of the body by eliminating old cells, unnecessary cells, and unhealthy cells, with the presence of pyknosis and occurrence of apoptotic bodies. Apoptosis exists during brain development, and there is a dynamic balance of proliferation and death of neural cells during nervous system development. Excessive or too little apoptosis of neural cells during embryonic development results in abnormal brain development[23]. Experimental results confirmed that cell apoptosis significantly increased in fetal rats with intrauterine growth restriction, which is probably the reason for deficits in brain development and the unfavorable prognosis of the nervous system[19, 23]. Taurine supplementation in pregnant rats reduced cell apoptosis in fetal rats with intrauterine growth restriction, exerting a protective effect on the brain.

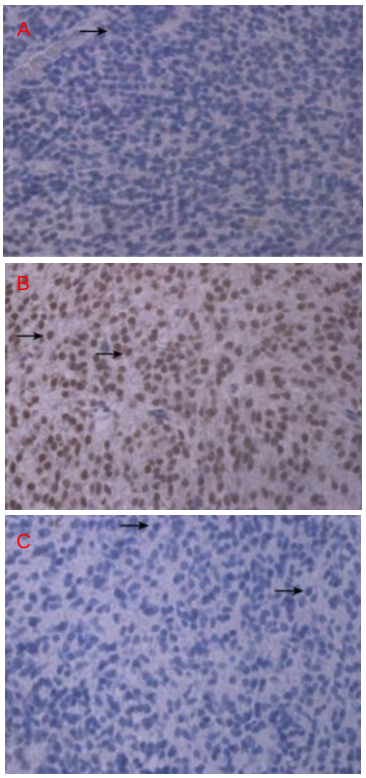

Figure 3 Caspase-3 expression in the cerebral cortex of fetal rats with intrauterine growth restriction (immunohistochemical staining, × 400).

Caspase-3 expression is mainly visible in nuclei (brown; arrows).

(A) Only a few caspase-3-positive cells are observed in the cerebral cortex of fetal rats of the control group.

(B) Caspase-3 expression significantly increases in the cerebral cortex of fetal rats in the model group.

(C) Taurine supplementation in pregnant rats noticeably diminishes caspase-3 expression in the cerebral cortex of fetal rats with intrauterine growth restriction.

Glial cell line-derived neurotrophic factor has a neurotrophic effect[24-25], and is a member of the transforming growth factor-β superfamily. Its neurotrophic effects were mediated by compound receptors of the glial cell line-derived neurotrophic factor-family receptor α1–4 and Ret tyrosine kinase (coded by proto-oncogene c-ret)[26-27]. The glial cell line-derived neurotrophic and its receptor complex bind to and stimulates autophosphorylation of Ret. The activated Ret subunit induced molecular downstream events through the phosphoinositide 3-kinases/protein kinase B and the mitogen-activated protein kinases/extracellular regulated kinase signal transduction pathway[28-29]. Thus, glial cell line-derived neurotrophic factor exerts its neurotrophic effect by regulating the apoptosis-related protein Bad and nuclear factor-kappa B to inhibit cell apoptosis and to promote neuronal survival, neuronal differentiation and regeneration of injured peripheral nerves[30], induction of neurogenesis in the infragranular layer of the dentate gyrus in the subventricular zone of the lateral ventricle and the hippocampus, maintenance of the development of the dopaminergic nigrostriatal system, and improvement of neuroethology[31]. Experimental results suggested that glial cell line-derived neurotrophic factor expression increased reactively to some extent in the cerebral cortex of fetal rats with intrauterine growth restriction, which would be an endogenous protective effect in the central nervous system. Nevertheless, this protective effect cannot compensate for the cell apoptosis induced by intrauterine growth restriction, but would result in neuronal loss, decreased neuron-specific enolase expression and cerebral dysgenesis[11].

There were no reports addressing whether taurine supplementation in pregnant rats affected glial cell line-derived neurotrophic factor expression in the brain tissues of fetal rats with intrauterine growth restriction. Experimental results demonstrated that glial cell line-derived neurotrophic factor expression increased in the cerebral cortex of fetal rats in the taurine group, indicating that taurine supplement in pregnant rats exerts a protective effect on the brain of fetal rats with intrauterine growth restriction probably through an increase in glial cell line-derived neurotrophic factor expression to some extent.

Caspases, or cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases, are a family of cysteine proteases that play an essential role in apoptosis. Caspase-3 is thought to be a key apoptotic “executioner” enzyme because its activation triggers the cascade of enzymatic events that culminates in the death of the cell[22]. Under normal conditions, caspase-3 exists as proenzyme in neural cells. Activated caspase-3 degrades key proteins in the nucleus, cytoplasm and cytoskeleton, finally resulting in cell apoptosis[32]. After brain injury, caspase-3 activation increases the permeability of endothelial cells and aggravates brain injury[33].

A previous study confirmed that acute hypoxia suppressed caspase-3 expression, and reduced cell apoptosis in the fetal ovine hippocampus to some extent[34]. Results from this study revealed that the number of caspase-3-positive cells significantly increased in brain tissues of fetal rats with intrauterine growth restriction, suggesting that caspase-3 participates in the onset mechanism of cell apoptosis in the brain of fetal rats with

intrauterine growth restriction. However, taurine supplementation in pregnant rats markedly diminished caspase-3 expression in the brain tissue of fetal rats with intrauterine growth restriction. Simultaneously, cell apoptosis noticeably reduced, indicating that the caspase-3 pathway is involved in the protective effect of taurine on the brain of fetal rats with intrauterine growth restriction.

Yu *et al* [35] verified that glial cell line-derived neurotrophic factor inhibited ischemia-induced cell apoptosis by suppressing caspase-3 activity. This study verified that taurine supplementation in pregnant rats promotes glial cell line-derived neurotrophic factor expression and reduces caspase-3 expression in the brain tissue of fetal rats with intrauterine growth restriction, which indicates that taurine supplementation involves the glial cell line-derived neurotrophic factor-caspase-3 signaling pathway to induce a protective effect on the brain.

In summary, taurine supplementation in pregnant rats inhibits cell apoptosis in fetal rats with intrauterine growth restriction through the glial cell line-derived neurotrophic factor-caspase-3 signaling pathway. A pilot study from our laboratory suggested that taurine supplementation in pregnant rats contributes to brain cell proliferation in fetal rats with intrauterine growth restriction[36]. Taken together, taurine supplementation in pregnant rats improves brain ultrastructure in fetal rats with intrauterine growth restriction and promotes brain development by promoting brain cell proliferation and reducing brain cell apoptosis[15-16]. This study provides theoretical support for the theory that taurine supplementation before delivery promotes brain development in fetal rats with intrauterine growth restriction.

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

Experiments were performed at the Central Laboratory of Bayi Children's Hospital, General Hospital of Beijing Military Command in China from January to June 2012.

Materials

A total of 15 healthy, clean, adult female Sprague-Dawley rats and eight male Sprague-Dawley rats weighing 250–300 g were purchased from the Vital River Laboratories, Beijing, China, license No. SCXX (Jing) 2007-0001. The rats were housed at 22 ± 6°C, 60–80% humidity, in a 12-hour light/dark cycle, and allowed free access to water. All procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[37].

Methods

Establishment of intrauterine growth restriction model

In accordance with previous studies[38-39], intrauterine growth restriction models were established by the low protein diet method. The female and male rats were housed in a cage at 2:1 every night. Vaginal secretions of female rats was taken the next morning and tested under a microscope. The day we detected sperm was considered the day of pregnancy. From the first day of pregnancy to parturition, the rats in the control group were administered standard food (20% (w/v) protein, 67% (w/v) carbohydrate and 13% (w/v) fat; Beijing HFK Bioscience Co., Ltd., Beijing, China). The rats in the model and taurine groups were given low protein food (8% (w/v) protein, 79% (w/v) carbohydrate and 13% (w/v) fate; Beijing HFK Bioscience Co., Ltd.). The amount of food was not restricted for each group. In the taurine group, 300 mg/kg taurine was added every day to the diet from the 12th day of pregnancy (0.4 g/bag, granules, lot No. 205-00115; Wako, Tokyo, Japan), until parturition.

Evaluation of intrauterine growth restriction

Within 6 hours after spontaneous delivery, the weight of filial rats was measured (accuracy to 0.01 g). The rats whose weight was below two standard deviations of the mean weight of filial rats from the control group were considered as fetal rats with intrauterine growth restriction[1, 38-39].

Collection of brain tissue from fetal rats

In the control group, two fetal rats were randomly selected from each brood of rats, except intrauterine growth restriction rats. In the taurine and model groups, two fetal rats with intrauterine growth restriction were randomly selected from each brood of rats and sacrificed. Brain tissue was weighed, fixed in 4% (w/v) paraformaldehyde, dehydrated in alcohol, and embedded in paraffin. Brain tissues were serially sliced into 4-μm-thick coronal sections at 2 mm from the anterior pole and 3 mm from the posterior pole of the brain[40].

TUNEL assay for cell apoptosis

In accordance with the TUNEL kit instructions (Boster,

Wuhan, Hubei Province, China), the sections were dewaxed, hydrated, treated with 3% (v/v) H_2O_2 to block endogenous peroxidase, digested with freshly diluted proteinase K solution at 37°C for 10 minutes, reacted with labeling solution in a wet box at 37°C for 2 hours, incubated in blocking buffer at room temperature for 30 minutes, and incubated with TUNEL reaction mixture at 37°C for 60 minutes. Subsequently, the sections were visualized with the 3,3′-diaminobenzidine kit (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China), counterstained with hematoxylin, dehydrated, permeabilized, and mounted. Five non-overlapped visual fields of each section were observed under a light microscope (× 400) (BX51; Olympus, Tokyo, Japan). Apoptotic cells were observed and counted.

Immunohistochemistry for glial cell line-derived neurotrophic factor and caspase-3 expression in brain tissue

Sections were dewaxed, hydrated, treated with 3% (v/v) H_2O_2 to block endogenous peroxidase activity, and immersed in 10 mmol/L citrate buffer solution (pH 6.0). Antigen retrieval was performed by heating sections for 15 minutes. After cooling at room temperature, the specimens were washed with PBS, incubated with rabbit anti-glial cell line-derived neurotrophic factor polyclonal antibody (1:200; Boster) and rabbit anti-caspase-3 polyclonal antibody (1:100; Boster) at 4°C overnight, and secondary antibody goat anti-rabbit IgG (1:500; Zhongshan Golden Bridge Biotechnology Co., Ltd.) at 37°C for 30 minutes, and incubated in horseradish peroxidase complex (Boster) at 37°C for 30 minutes. Three PBS washes were performed between each step. The sections were visualized with 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted. PBS served as a negative control instead of primary antibody. Five non-overlapped visual fields of each section were observed under a × 400 microscope. Positive cells were observed and counted.

Statistical analysis

Data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Measurement data with normal distribution were expressed as mean ± SD. The homogeneity test for variance was performed using Levene's test. One-way analysis of variance was used if homogenous variance was present. Paired comparison was performed using the Student-Newman-Keuls test. The Kruskal- Wallis rank sum test was used if heterogeneity of variance was present. Paired comparison was performed using Tamhane's test. A value of $P < 0.01$ was considered statistically significant.

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