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Effects of ganoderic acids on epileptiform discharge hippocampal neurons: insights from alterations of BDNF, TRPC3 and apoptosis

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Recently, *Ganoderma lucidum* spores (GLS) have shown anti-epileptic effects. However, there are no reports on the anti-epileptic effects of its chemical constituents ganoderic acids (GAs), and more research is needed to better understand the mechanism of GLS activity. In this work, rat primary hippocampal neurons in an *in vitro* model were used to assess the intervention effects of GAs on epileptiform discharge hippocampal neurons and expression of both BDNF and TRPC3, with the aid of immunofluorescence, MTT method and flow cytometry. It was found that BDNF and TRPC3 are expressed in all cells and were mainly localized in the cytoplasm. The fluorescence intensities of BDNF and TRPC3 in GAs groups were higher than those of normal control and model groups, especially at 80 μg/ml (P<0.05). The apoptosis rate of neurons was inversely proportional to BDNF and TRPC3 changes (P<0.01). Therefore, BDNF and TRPC3 should be involved in the occurrence and development of epilepsy. GAs might indirectly inhibit mossy fiber sprouting and adjust the synaptic reconstructions by promoting the expression of BDNF and TRPC3. Besides, GAs could exert a protective effect on hippocampal neurons by promoting neuronal survival and the recovery of injured neurons.

1. Introduction

Epilepsy, a cerebral dysfunction syndrome characterized by transient but recurrent disturbances, is one of the most common neurological diseases, associated with over 65 million patients worldwide (Chang and Lowenstein 2003; Silberberg et al. 2015). Most epileptic cases come from excessive and abnormal synchronization of the brain, with recurrent seizures and impairment/loss of consciousness, but the exact pathogenesis is unknown (Goldberg and Coulter 2013). In recent times, neurosurgery became an option for people with partial seizures, nonetheless, daily anti-epileptic drugs (AED) play a pivotal role in the treatment of epilepsy (Gleichgerrcht et al. 2015; Mintzer et al. 2015). Unfortunately, all available AEDs exhibit issues with central nervous system (CNS) tolerability, that might affect the quality of life and contribute to treatment failure (Loscher et al. 2013; Schmidt 2009). Hence, it is valuable to discover new ingredients with a submaximal response and minimizing side effects.

Herbal remedies have become a hot topic for treating patients with neurologic or psychiatric complaints (Tyagi and Delanty 2003). Previous studies in our laboratory have revealed that *Ganoderma lucidum* spores (GLS) could decrease hippocampal epileptiform activities, via attenuating the apoptosis induced by epilepsy and regulating the expression of proteins related to neuron development (e.g. CaMKIIα, N-cadherin and neurotrophin-4) (Wang et al. 2014, 2013). Among the bio-active ingredients of GLS, ganoderic acids (GAs, oxygenated lanostane-type triterpenoid) are major chemical constituents responsible for many pharmacological immunomodulatory, antitumor and antiviral effects (Boh et al. 2007; Sanodiya et al. 2009; Shiao 2003; Wachtel-Galor et al. 2011; Zhu et al. 2015). However, there are no reports on the anti-epileptic effect of ganoderic acids, and more research is needed to better understand the mechanism of GLS activity.

The transient receptor potential (TRP) superfamily is one of the largest ion channel families, which has an important regulatory role in sensory signal initiation (Clapham 2003; Voets et al. 2005). In mammals, the TRP superfamily comprises six subfamilies: TRPC (TRP-canonical), TRPV (TRP-vanilloid), TRPM (TRP-melastatin), TRPML (TRP-mucolipin), TRPP (TRP-polycystin) and TRPA (TRP-ankyrin), with some structural similarity to each other (Venkatachalam and Montell 2007). In general, seven subtypes of TRPCs (TRPC1-7) are all involved in Ca^{2+} homeostasis, and known as Ca^{2+}/cation selective channels (Huang et al. 2011). Recently, evidence has been provided that the TRPC-dependent calcium fluxes are crucial to the neuronal protection effect of the brain-derived neurotrophic factor (BDNF, a potent target-derived pro-survival protein) (Jia et al. 2007; Li et al. 2005; Sossin and Barker 2007). In cerebellar granule neurons (CGN), TRPC3 and 6 channels contribute to the maintenance of Ca^{2+} elevation and growth-cone turning induced by BDNF, and more importantly, disruption of their function reduces CGN survival *in vivo* (Jia et al. 2007; Li et al. 2005). Besides, TRPC3 is highly enriched in the CNS neurons, and cation influx through TRPC3 should parallel those of neurotrophin receptor TrkB and phospholipase C (PLC) (Leal et al. 2014; Li et al. 1999).

So far, no studies have been performed to detect the neuroprotective effect of GAs and correlative effects on the expression of TRPC3. In this work, the above issues will be addressed using rat primary hippocampal neurons in an *in vitro* model: 1) Immunofluorescent staining of neuron specific enolase (NSE) was used to assess morphology and purity of cultured hippocampal neurons; 2) a sterile method for GAs generation was studied to ascertain the optimal concentration gradients of GAs in test models; 3) a confocal laser scanning (CLS) method was adopted to elucidate the expression of BDNF and TRPC3 in neurons of GAs and control treated neurons; 4) flow cytometry was used to detect the percentage of apoptosis cells. We anticipated that the data prove valuable for understanding the correlation of anti-epilepsy effect and a potential interaction mechanism for GAs.

2.1. Investigations and results

2.1. Morphology observation

Initially, the cultured hippocampal neurons were round or oval, presented as single or several cells in a cluster dispersedly. But led soon, live cells were adherent monolayer growth, with clear cell

borders. After 24 hours, some of the cells stretched out neurites, with spindle or irregular shape. Along with time, the protrusions of the neurons increased gradually (Fig. 1). After 3 days, the number of the neurons increased, associated with the connected network of neurites. Neurons had high three-dimensional quality and refraction (Fig. 1). Five days later, the haloes of the neurons were obvious, with bigger somas. Neurites were dense, thick and long and knitted together (Fig. 1). At day 9, the neurons got mature and aggregated into clumps, with the unevenly distribution. At this time, it was hard to identify a single neuron (Fig. 1).

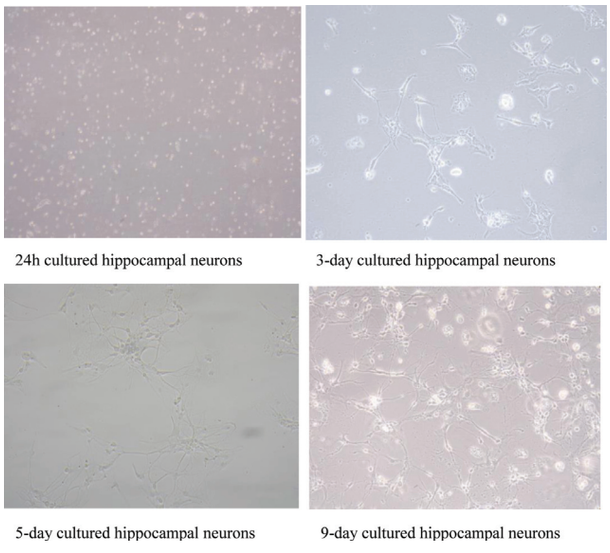

Fig. 1: Hippocampal neurons in various days (×200)

2.2. Identification and purity of hippocampal neurons

Hippocampal neurons were detected by NSE immunofluorescence staining. It was found that the mature bodies are plump, triangular or round, at the same time, their neurites were dense, thick and interweaved into a network (Fig. 2). Cytoplasm and neurites were colored in green, revealing the presence of NSE. Conversely, the nuclei were stainless. At day 9, the hippocampal neurons differentiated completely, and the purity of hippocampal neurons was higher than 96 % (Table 1). At day 11 and 14, the hippocampal neurons grew well, in contrast to the situation of day 9, without obvious change of purity. Hence, these neurons could be used in the modeling of epileptiform discharge hippocampal neurons.

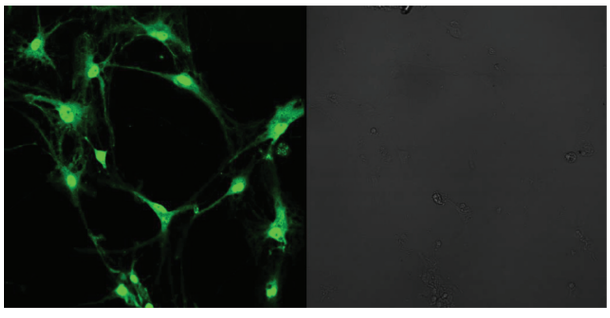

Fig. 2: Immunofluorescence stain of NSE in hippocampal neurons (×400)

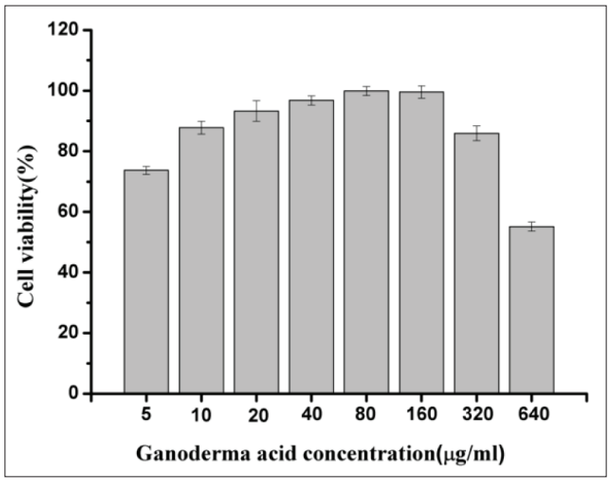

Fig. 3: Determinate toxicity of ganoderic acid against hippocampal neurons

2.3. MTT results

The toxic effects of GAs on the hippocampal neurons included the increase of granules in the cytoplasm, decrease of transparency, cracking of neurites, deforming and detachment of the cells. With the decline of GAs concentration, the toxic effects gradually weakened, with the increasing of survival neurons. According to the pharmaceutical dilution, the most suitable drug concentration (optimal non-toxic) of GAs on hippocampal neurons was calculated as 80 μg/ml (Fig. 3). More toxic effects were exerted by higher concentrations, such as 640 μg/ml. Therefore, 20, 80 and 320 μg/ml were adopted for the subsequent experiments.

2.4. BDNF and TRPC3 expression

The expression levels of BDNF and TRPC-3 were reflected by immunofluorescence staining results: in each experiment group,

Table 2: BDNF fluorescence intensity in different groups ()

Normal control group	Model group	GAs groups		
		Low concentration	Moderate concentration	High concentration
0.609±0.073	0.679±0.063*	0.756±0.059#	0.916±0.063★	0.850±0.065◆

*P<0.05 *vs* normal control group, #P<0.05 *vs* model group, ★, ◆P<0.01 *vs* other concentrations of GAs groups

Table 1: Purity of hippocampal neurons (%)

Time (day)	5	7	9	11	14
Hippocampal neurons	33.13±11.18	63.03±17.73	83.15±23.85	67.09±18.81	80.93±17.78
Glial cells	4.50±2.07	5.16±1.66	3.06±1.41	2.71±1.33	3.06±1.15
Purity (%)	87.9±5.0	91.0±3.0	96.0±3.0	96.0±1.0	96.0±1.7

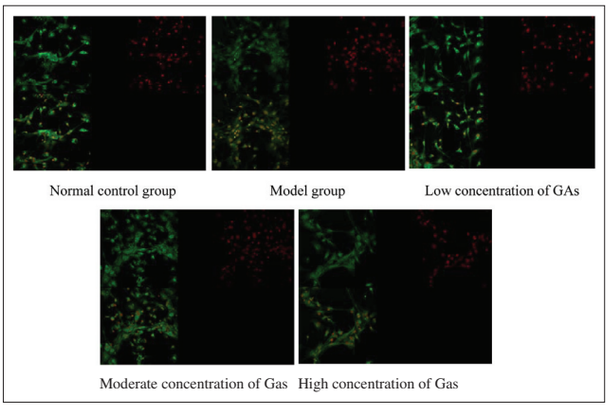

Fig. 4: BDNF expression in neurons

Table 3: TRPC3 fluorescence intensity in different groups ()

Normal control group	Model group	GAs groups		
		Low concentration	Moderate concentration	High concentration
0.662±0.050	0.767±0.091*	0.850±0.065#	0.925±0.065★	0.913±0.088◆

*P<0.05 *vs* normal control group, #P<0.05 *vs* model group, ★, ◆P<0.01 *vs* other concentrations of GAs group

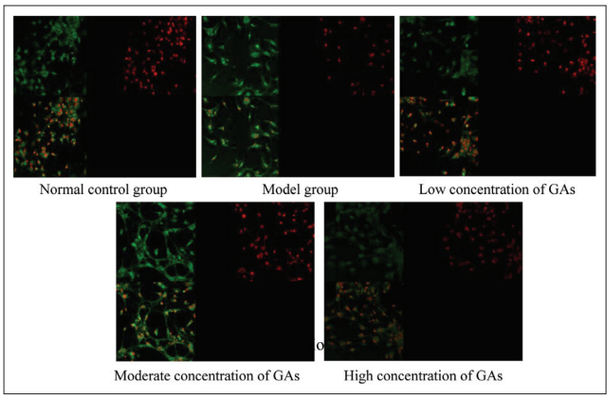

Fig. 5: TRPC3 expression in neurons

Table 4: Apoptosis rate in different groups ()

Normal control group	Model group	GAs groups		
		Low concentration	Moderate concentration	High concentration
8.600±2.420	54.40±0.080*	25.65±0.405#	19.85±6.125★	32.25±0.845◆

*P<0.05 *vs* normal control group, #P<0.05 *vs* model group, ★, ◆P<0.01 *vs* other concentrations of GAs groups

BDNF and TRPC-3 were detected and mainly located in the cytoplasm; in the model group, the expression levels of BDNF and TRPC-3 were higher than those of the normal control group ($P < 0.05$); in GAs groups (various concentrations of ganoderic acids), the two levels were also higher than those of normal group ($P < 0.05$). The differences were statistically significant ($P < 0.05$), especially at the concentration of 80 μg/ml (Tables 2 and 3, Figs. 4 and 5).

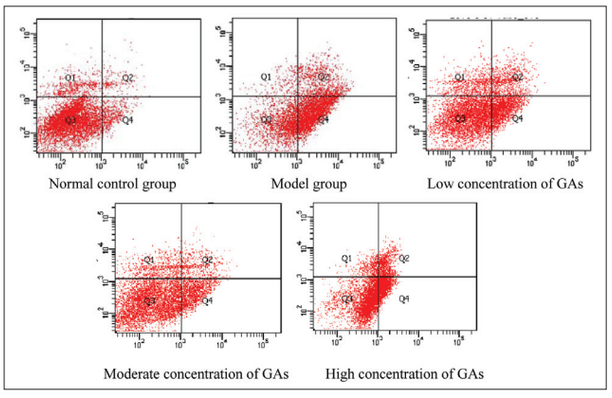

Fig. 6: Apoptosis rate in each group

2.5. Apoptosis rates

The apoptosis rate in the model group was higher than that in the normal control group ($P < 0.01$), the values of GAs groups (various concentrations of ganoderic acids) were lower than that of the model group ($P < 0.01$). The differences were statistically significant ($P < 0.05$), especially at the concentration of 80 μg/ml (Table 4 and Fig. 6).

3. Discussion

Although great efforts have been made to the development of anti-epileptic drugs and a variety of new epilepsy drug were constantly explored, still one third patients with epilepsy are not sensitive to the drug therapy (Perucca et al. 2007). At the same time, it was found that many antiepileptic drugs have side effects like cognitive dysfunction, neurological side effects, and recurrent epilepsy (Schmitz 2006). Current antiepileptic drugs can only control the seizures, with no effect on pathological changes and the process of epilepsy (Rogawski and Loscher 2004). *Ganoderma lucidum* is a kind of precious medicinal fungi, with obvious anti-epileptic effects, containing polysaccharides and triterpenoids as main effective components. Ganoderic acids (GAs), triterpene compounds, are primary chemical constituents of *Ganoderma lucidum* spores (GLS), with important biological activities, but their anti-epileptic effect and mechanism of action are still not clear (Luo and Lin 2002; Wang et al. 2014, 2013). Therefore, in this work, rat primary hippocampal neurons in an *in vitro* model were used to explore the role and potential mechanism of GAs against epilepsy, by analyzing cell apoptosis and BDNF / TRPC3 expression.

Regarding epilepsy, hippocampus is the most sensitive part of the central nervous system (CNS), and its transformation has often been studied. Our experiments revealed that epileptiform discharge hippocampal neurons can be caused by Mg^{2+}-free treatment, with increased release of BDNF (Table 2 and Fig. 4). This means that BDNF should play an important role in the pathophysiological changes leading to epilepsy (Binder et al. 2001). Mounting evidence indicates that BDNF can reduce the excitability of neurons, prevent or slow the seizures and seizure-induced neuronal damage. Besides, BDNF can also stimulate the growth of neurons, which have selective vulnerability induced by status epilepticus (SE). Above all, BDNF facilitates the translocation of dentate gyrus neurons, dendrites of granulosa cells, as well as the growth of mossy fibers (Hagihara et al. 2005). And it has been revealed that mossy fiber sprouting plays a key role in pathogenesis of temporal lobe epilepsy (Buckmaster 2014).

Compared with the model group (epileptic neurons), the BDNF expression of surviving neurons in GAs groups (epileptic neurons treated with 20/80/320 μg/ml ganoderic acids) was increased, with enhanced immune response ($P < 0.05$) (Table 2 and Fig. 4). This indicates that GAs can promote the expression of BDNF in epileptiform discharge hippocampal neurons, being important to protect

cerebral neurons and promote the recovery of injured neurons. The anti-epileptic mechanism of GAs may be attributed to the following route: GAs enhance the upregulated expression of BDNF in the hippocampus, then promote the expression of calcium binding protein (CaBP), excrete excessive Ca^{2+} within the cells, prevent the internal flow of extracellular Ca^{2+} and release of intracellular Ca^{2+}, finally maintain intracellular Ca^{2+} concentration and environmental homeostasis (Goldberg and Coulter 2013; Loscher et al. 2013). However, more specific action pathways and interaction mechanism of GAs remain to be further research. Numerous studies have demonstrated that BDNF can delay the attack time of the seizures, not only reduce the severity of seizure, but also inhibit mossy fiber sprouting by changing the expression of various TRPC subtypes (Jia et al. 2007; Li et al. 2005). Combined with our experimental results, in contrast to the model group, the TRPC3 expression of surviving neurons in GAs groups (epileptic neurons treated with 20/80/320 μg/ml ganoderic acids) was increased, with enhanced immune response ($P < 0.05$) (Table 3 and Fig. 5). This suggests that TRPC3 may participate in the process of development and plasticity of neurons, and BDNF may mediate the process of hippocampal synaptic reconstruction introduced by TRPC3. Therefore, the BDNF-TRPC channel is expected to become a new target for valid treatment strategy.

Seizures can cause the apoptosis of neurons. Our experimental results indicated that apoptotic hippocampal neurons were significantly reduced with the treatment of GAs (20/80/320 μg/ml, Table 4 and Fig. 6). GAs can inhibit the apoptosis of epileptiform discharge hippocampal neurons and protect brain tissue, by inhibiting the formation of apoptosis stimulating factors or promoting the protection effects of neurotrophic factors on apoptotic cells, as well as influencing the expression of genes related in apoptosis. However, there is still some research needed to explore the mechanism of this action.

In conclusion, GAs may indirectly inhibit mossy fiber sprouting and adjust the synaptic reconstructions by promoting the expression of BDNF and TRPC3. Then, GAs may exert a protective effect on hippocampal neurons by promoting neuronal survival and the recovery of injured neurons. Further studies on the therapeutic use of GAs are needed to support this point of view.

4. Experimental

4.1. Animals

Newborn Wistar rats (within 24 hours) were purchased from Animal Center of Jiamusi University, undergoing operations with the guideline of animal ethical committee in Jiamusi University.

4.2. Culture of hippocampal neurons

Hippocampal tissues from newborn Wistar rats were harvested using conventional methods (Su et al. 2011; Wang et al. 2013). 4, 2 and 0.2 ml cell suspensions (a density of 5 x 10^5/ml) were transferred into 25 cm culture plate, each well of 6- and 96-well plates. Cultures were incubated at 37 °C with 5% CO_2 for 24 h. When the cultured cells grew against the wall, the whole cultured medium [Neurobasal medium (Cat. No. 21103049, Gibco), 2% B27 supplement (Cat. No. 17504044, Gibco), 0.5 mmol/L glutamine and 10% FBS] was replaced by nutrient maintaining medium [Neurobasal medium, 2% B27 supplement and 0.5 mmol/L glutamine]. Half amount of the volume of medium was replaced every other day. In 4th day, 10 μmol/L cytarabine was added into each culture flask. After 24-hour culture, the whole maintaining medium was entirely renovated.

4.3. Identification of hippocampal neurons

Hippocampal neurons were identified by detection of NSE (a useful marker for neuronal cells) with antibodies, via immunofluorescence technique (Wang et al. 2013). Briefly, 9-day-cultured hippocampal neurons were rinsed 3 times using 0.01 mol PBS (5 min each time), and then incubated with anti-NSE antibody (Cat. No. BA0535, Wuhan Boster Bio-Engineering Ltd, China) and secondary antibody labeled with FITC (Cat. No. BA1105, Wuhan Boster Bio-Engineering Ltd, China). Microscope observation of fluorescence was performed to obtain pictures.

4.4. Purity of hippocampal neurons

At a density of 5×10^5/ml, hippocampal neurons were cultured, and then four culture flasks were chosen for day 5, 7, 9, 11 and 14 to calculate the purity of hippocampal neurons, using the formula: number of hippocampal neurons / (number of hippocampal neurons + number of glial cells) (Wang et al. 2013). The numbers of hippocampal neurons and glial cells were counted under a 100× microscope, within 10 randomly selected visual fields.

4.5. Modeling of epileptiform discharge hippocampal neurons

An epileptic model of hippocampal neurons was built by the conventional method (Sliva et al. 2012; Wang et al. 2013). Details of setup were in agreement with the reference (Wang et al. 2013). Briefly, at the 9th day, nutrient medium was changed into Mg^{2+} free extracellular medium (145 mmol NaCl, 2.5 mmol KCl, 2 mmol $CaCl_2$, 10 mmol HEPES, 10 mmol glucose, 0.002 mmol glycine, pH 7.2, 290+10 mOsm) and cultured for 3 h. Then, this medium was replaced with the normal extracellular medium (including 1 mmol $MgCl_2$) and incubated for a further 6 h.

4.6. Cytotoxicity assays

An optimized concentration of GAs to hippocampal neurons was determined by MTT assay (Cao et al. 2003). Briefly, cells were plated in 96-well culture plates (5×10^4 cells/well) separately. After 9 days incubation, neurons were treated with GAs (5, 10, 20, 40, 80, 160, 320, 640 μg/ml, eight wells per concentration) in a CO_2 incubator at 37 °C, for 24 h. Besides, normal cells were used as a control group. MTT solution (5 mg/mL) was then added to each well. After 4 h incubation, the formazan precipitate was dissolved in 200 μL dimethyl sulfoxide, and then the absorbance was measured in an ELISA reader (Thermo Molecular Devices Co., Union City, CA, USA) at 490 nm. The experiment was repeated three times. It was found that the concentration of GAs should be divided into 20, 80 and 320 ug/ml.

4.7. Analysis of BDNF and TRPC3 expression

Hippocampal neurons cultured for 9 days were used and randomly divided into the following groups: 1) normal control group, the medium was changed into normal extracellular medium and cultured for 3 h, then recovered to the normal culture for a further 3 h; 2) model of epileptiform discharge hippocampal neurons group (model group), cells were treated with Mg^{2+} free extracellular medium for 3 h, then cultured with normal medium for a further 3 h; 3) GAs groups, cells were treated with Mg^{2+} free extracellular medium for 3 h, then cultured with normal medium containing GAs for a further 3 h. Based on the results of above section, the concentration of GAs was divided into low, medium, and high (20, 80 and 320 μg/ml), respectively.

The conventional immunofluorescent techniques were used, and images were taken by the confocal laser scanning (CLS) method (Wang et al. 2014, 2013). Briefly, the samples were labeled and incubated with a rabbit-anti-BDNF BAB and rabbit-anti-TRPC3 BAB (Cat. No. BA0565-1 and BA3393, Wuhan Boster Bio-Engineering Ltd, China), associated with secondary antibody labeled with FITC. For each group, fifteen neurons were chosen randomly, and Image-Pro Plus software (Media Cybernetics, Silver Spring, MA, USA) was used to assay the absolute value of fluorescence intensity (O'Mahony et al. 2005).

4.8. Analysis of apoptosis

Each sample was washed twice with 0.01 M PBS, and made into a single-cell suspension with a density of 1×10^4/ml. Then, the suspension was taken out and centrifuged at 1000 rounds per minute at 4 °C for 5 min, and the supernatant was discarded. PBS was added and shaken gently to resuspend the cells. 100 μL cell suspension was treated with 5 μL Annexin V-PE and 5 μL 7AAD (Cat. No. KTK102-020, Beijing Korad Biotechnology Co., Ltd), with being shielded from light at room temperature for 15 min. Apoptotic cells were analyzed with a FACSCalibur® flow cytometer (BD Biosciences, Burlington, MA, USA) (Bilsland et al. 2006).

4.9. Statistical analysis

Data were expressed as mean±standard deviation (n = 3). The significance of difference was calculated by one-way ANOVA (SPSS13.0) and values $p < 0.05$ were considered to be significant.

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Conflicts of interest: None declared.

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