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Cyclic adenosine phosphate improves functional recovery after spinal cord injury via activating unfolded protein response

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The current study aimed to evaluate the role and underlying mechanism of cyclic adenosine phosphate (cAMP) on the functional recovery of spinal cord injury (SCI). Basso, Beattie and Bresnahan (BBB) scoring and inclined plane test indicated that cAMP treatment improved the functional recovery of SCI rats. Real time PCR and western blot analysis showed the mRNA and protein levels of IRE1, PERK, and ATF6 were increased in the SCI rats than those of sham control. However, higher levels of IRE1, PERK, and ATF6 were indicated after cAMP treatment. Meanwhile, more apoptotic cells were observed in the SCI rats, as evidenced by TUNEL staining and increased expression of GRP78, CHOP, and caspase12. In contrast, the expression of GRP78, CHOP, and caspase12 was decreased in SCI rats after cAMP treatment. In summary, we showed novel data that cAMP reduced cell apoptosis and functional recover after SCI mainly via activating UPR.

1. Introduction

Spinal cord injury (SCI) results in invalidating locomotor impairment and leads to life-long disability for the patients (He et al. 2017; Wang et al. 2017). SCI often leads to an enormous social and economic impact via inflammation, hypoxia, excitotoxicity, disruption of blood brain barrier, and ischemia (Fassbender et al. 2012; Fan et al. 2015). Hence, it is of great importance to explore the underlying mechanism by which the functional deficits after SCI are regulated.

The endoplasmic reticulum (ER) is the intracellular organelle that plays a key role in the maintenance of cellular homeostasis via modulating the synthesis and proper folding of proteins (He et al. 2017; He et al. 2017). Abnormal disruption of ER function and activation of unfolded protein response (UPR) are widely identified in the progression of various diseases (Lee et al. 2014; He et al. 2017). It is suggested that motor recovery after SCI is activated by increasing the unfolded protein response (Valenzuela et al. 2012). Dl-3-n-butylphthalide is shown to improve functional recovery in SCI rats by suppressing ERS-induced apoptosis (He et al. 2017). These data indicate that URP and ERS are key regulators in the progression of SCI.

Adenosine is one of the important inhibitory neurotransmitters in the central nervous system (Soty et al. 2016). Increasing evidence has shown that cyclic adenosine phosphate (cAMP) can promote nerve regeneration after SCI, thereby exerting protective effects on ischemia, hypoxia and traumatic brain injury (Liu et al. 2015; He et al. 2016). However, whether cAMP prevents SCI via URP has been poorly studied. In this study, by intralipbal injection of double butylated cyclic adenosine monophosphate (dibutyryl-cAMP), the spinal dorsal hemisection injury model was used to observe the role of cAMP after SCI, which may shed light on a new strategy for the clinical treatment of spinal cord injury.

2. Investigations and results

2.1. cAMP improves motor behavioral function in rats with SCI

First, we evaluated whether cAMP protects rats from SCI. As shown in Fig. 1A, BBB scoring indicated that the movement capacity was significantly improved by cAMP treatment on day 3, 7, 14, 21 days than those of SCI group. Inclined plane test showed that the hind limb strength was much weaker in the SCI group, but the hind limb strength was increased after cAMP treatment for 3, 7, 14, 21 days (Fig. 1B).

2.2. cAMP activated UPR in the spinal cord

IRE1 (type I ER transmembrane protein kinase), PERK (pancreatic eIF-2 kinase) and ATF6 (activating transcription factor 6) are key regulators of UPR, which can effectively amplify stress signals and initiate signal transduction. UPR is associated with the occurrence of many diseases. Therefore, understanding of the molecular mechanism of UPR is of positive significance for studying the mechanism of SCI and seeking new therapeutic targets. Real time PCR showed that the mRNA levels of IRE1, PERK, and ATF6 was much higher in the spinal cord tissues of SCI group than those of sham control (Fig. 2A). Interestingly, we found that cAMP treatment significantly increased the mRNA levels of IRE1, PERK, and ATF6 (Fig. 2A). Additionally, we explored the protein levels of IRE1, PERK, and ATF6 after cAMP treatment. In line with mRNA levels, the expression of IRE1, PERK, and ATF6 was higher in the SCI group than in the sham group (Fig. 2B). After cAMP treatment for 7 days, the protein expression of IRE1, PERK, and ATF6 was significantly increased (Fig. 2B). These data suggested that cAMP activated UPR after SCI.

2.3. cAMP reduced cell apoptosis in the spinal cord

Furthermore, we examined whether cAMP reduced spinal cord cell apoptosis. TUNEL staining demonstrated the enhancement of cell apoptosis in the SCI group compared to control (Fig. 3A). In contrast, after cAMP treatment, apoptotic cells were obviously decreased in the spinal cord tissues (Fig. 3A). We also explored the three major proteins that were related to ERS-induced cell apoptosis, namely GRP78, CHOP, and caspase12. Our data showed that the protein levels of GRP78, CHOP, and caspase12 were significantly enhanced in the SCI group (Fig. 3B) while their expression was much lower after cAMP treatment (Fig. 3B).

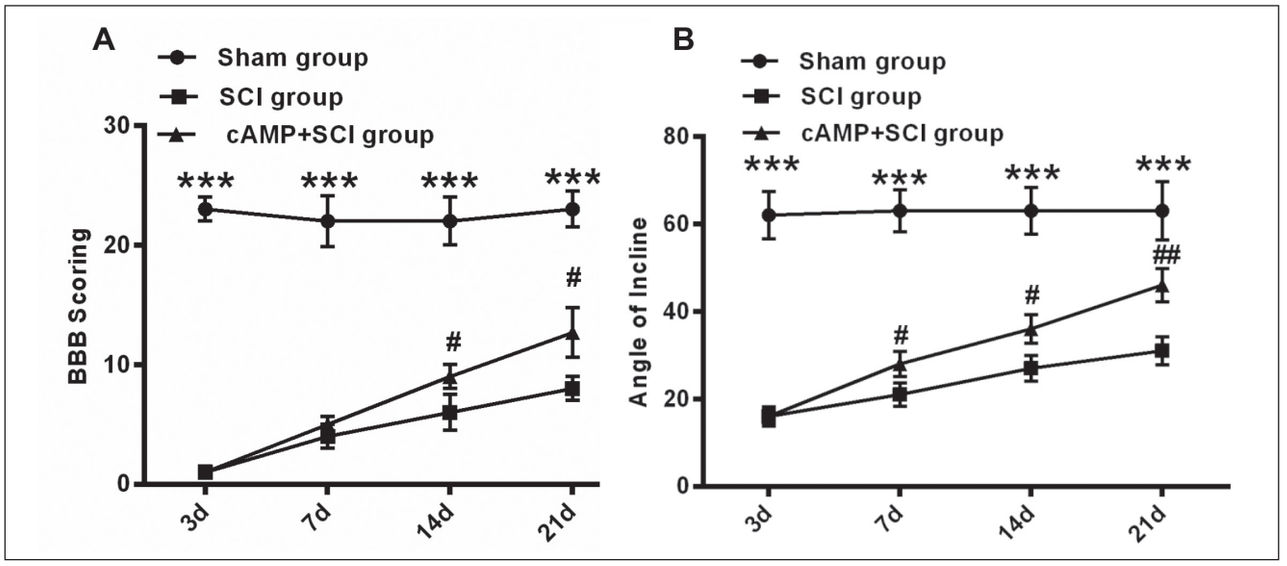

Fig. 1: cAMP improves motor behavioral function in rats with SCI. (A) BBB scoring indicated that the movement capacity was significantly improved by cAMP treatment. (B) Inclined plane test showed that the hind limb strength was much weaker in the SCI group, but the hind limb strength was increased after cAMP treatment for 3, 7, 14, 21 days. *p<0.05, **p<0.01, ***p<0.001, as indicated.

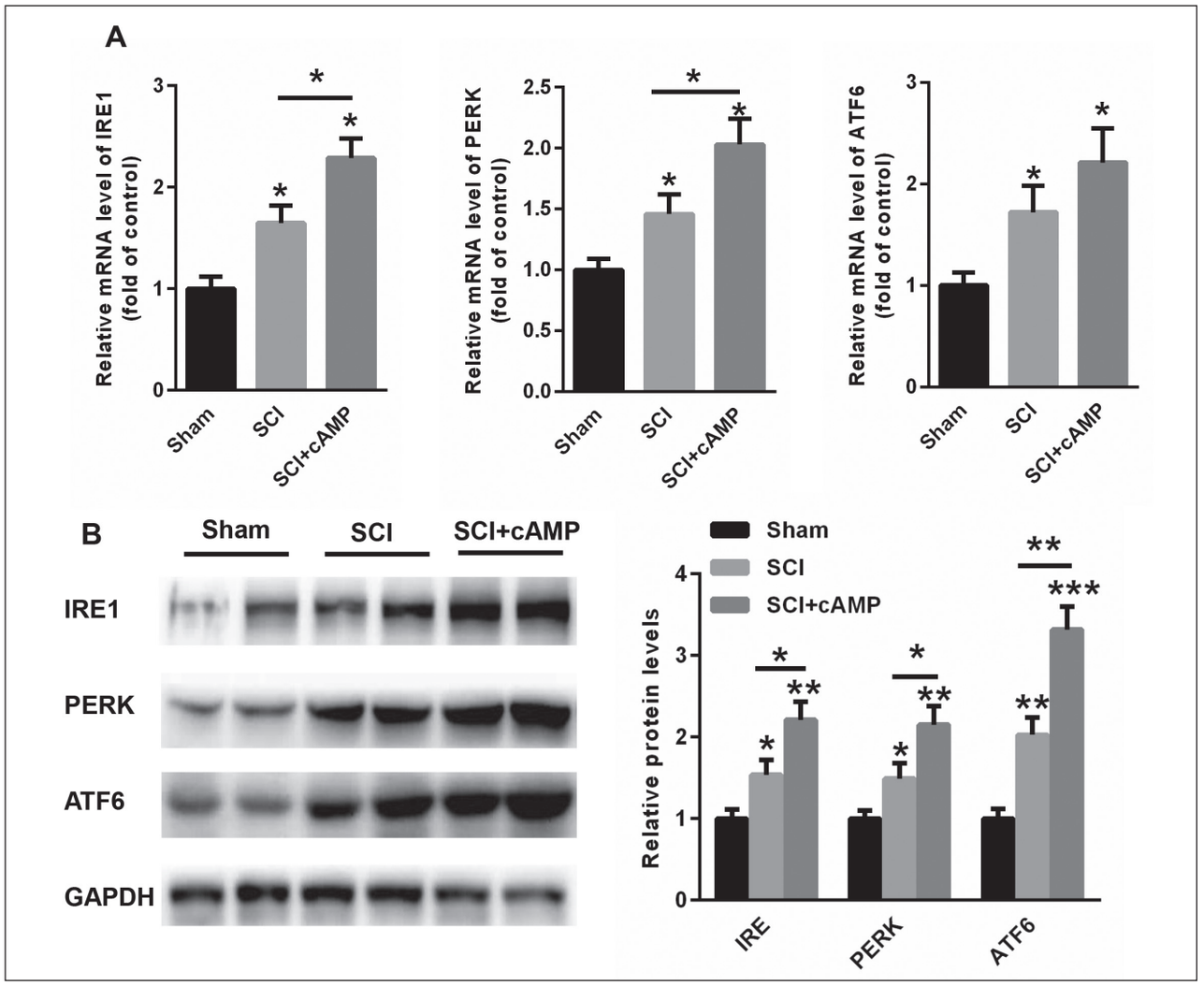

Fig. 2: cAMP activated UPR in the spinal cord. (A) The mRNA levels of IRE1, PERK, and ATF6 were evaluated using real time PCR. (B) The protein levels of IRE1, PERK, and ATF6 were analyzed using western blot analysis. *p<0.05, **p<0.01, ***p<0.001, as indicated.

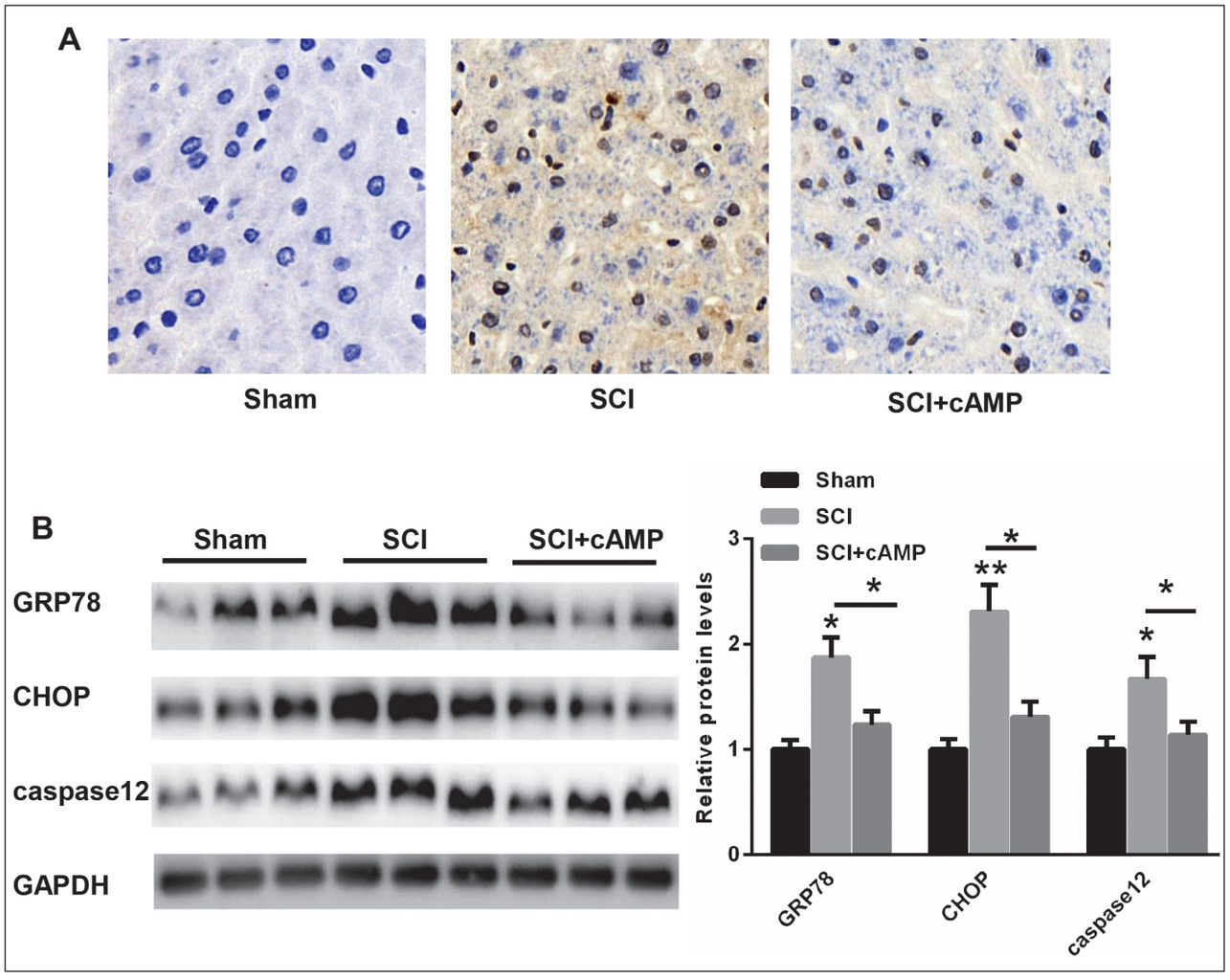

Fig. 3: cAMP reduced cell apoptosis in the spinal cord. (A) TUNEL staining demonstrated that cAMP treatment reduced cell apoptosis. (B) Western blot assay showed that cAMP treatment reduced the protein levels of GRP78, CHOP, and caspase12. *p<0.05, **p<0.01, ***p<0.001, as indicated.

3. Discussion

Protein-folding stress at the endoplasmic reticulum (ER) plays a key role in the development of spinal cord injury (Matsuyama et al. 2014; Liu et al. 2015). It is found that the recovery of spinal cord function involves the correction of protein misfolding and aggregation (Ohri et al. 2012). Hence, in the current study, we mainly focused on the unfolded protein response (UPR), an adaptive reaction against ER stress.

After SCI, second messengers, including cAMP, are reported to promote axonal regeneration (Ohri et al. 2011, 2014). For instance, activation of cAMP signaling is found to promote axonal regeneration in spinal cord-lesioned zebrafish (Bhatt et al. 2004). In addition, upregulation of endogenous cAMP is demonstrated to enhance axonal regeneration and increases functional recovery (Neumann et al. 2002). However, whether cAMP is involved in the UPR has been poorly understood.

Our data show that UPR, an adaptive reaction of ER stress, is activated in the SCI rats. It is well known that UPR restores cellular proteostasis or induces apoptosis of damaged cells (Penas et al. 2007; Saraswat Ohri et al. 2018). Studies have indicated that UPR is initiated by activation of several proteins, including IRE1α, PERK and ATF6 (Wu et al. 2016, 2018). IRE1α results in the production of an active transcription factor, XBP1s, which regulates protein folding, secretion, phospholipid biosynthesis and ER-associated protein degradation (Zhang et al. 2014). And PERK suppresses downstream protein translation *via* the phosphorylation of eIF2α, thereby modulating protein metabolism, folding and autophagy (Wu et al. 2018). Interestingly, our data show that cAMP treatment could further enhance the translation of expression of UPR proteins, including IRE1α, PERK and ATF6. Meanwhile, BBB scoring and inclined plane test indicated that cAMP treatment could significantly improve the functional recovery of spinal cord. These data show that cAMP-induced the improvement of spinal cord function is partially mediated via UPR.

The relationship of cell apoptosis and UPR is controversial (Penas et al. 2007). Some studies suggested UPR pathway to primarily enhance cell survival (Cooper et al. 2017; Eigner et al. 2017), while others suggest that UPR leads to cell death including pro-apoptotic pathways (Haney et al. 2017; Lee et al. 2017). Here, we demonstrated increased cell apoptosis in SCI, as indicated by increased expression of pro-apoptosis proteins, including GRP78, CHOP, and caspase12. In contrast, cell apoptosis was decreased after cAMP treatment, which is accompanied by further activation of UPR. We propose that enhanced UPR-activity was associated with increased flexibility to cope with ER stress, thereby exerting the pro-survival of cells in the spinal cord tissues. These findings further validated the protective role of cAMP in the functional recovery of SCI rats.

In summary, we showed novel data that cAMP reduced cell apoptosis and functional recover after SCI mainly via activating UPR.

4. Experimental

4.1. Establishment of a rat SCI model

A total of 56 specific-pathogen free male Sprague Dawley rats (weight 220-250 g, 6-8 weeks old) were purchased from Shanghai Silaike Experimental Animal Limited

Liability Company (Shanghai, China). Rats were maintained in the animal experimental center of the First Affiliated Hospital of Harbin Medical University and four animals were housed per each cage with a 12-h light/dark cycle. Room temperature was maintained at 23±1 ˚C, humidity was maintained at ~60 % and all rats had free access to food and water. Rats were randomly divided into three groups: sham, an SCI and an cAMP (60-92-4, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany)+SCI group. Before use, cAMP was diluted into 30 mM.

A rat SCI model was established as previously described (Chen et al. 2017). Rats in the sham surgery group underwent all aspects of the surgery except for contusion thoracic injury. Following 8 h fasting, rats were anesthetized via intraperitoneal injection with 10% chloral hydrate (400 mg/kg), according to a previous report (Vachon et al. 2000). Subsequently, rats were placed in a prone position and an incision (~2.5 cm in length) was made in the middle of the back. Skin was cut layer by layer and the T8 to T10 vertebral plates were exposed. Total laminectomy was performed for the T9 vertebral plate to expose the spinal dura mater. T8 and T10 spinous processes were fixated using forceps. A Kirschner wire (10 g) was inserted into the catheter that was inserted into the aorta with a weight, which fell freely from a 25-mm height. Following this, a semicircular slice (4x2 mm) made from thin plastic was hit, and the wire was immediately removed, resulting in incomplete injury of the rat spinal cord. The incision was sutured layer by layer. Following the strike, rats exhibiting a tail-wagging reflex, retraction flutter in the lower limbs and body, and flaccid paralysis in the lower limbs in an awake state represented successful model construction. For cAMP treatment, the rats were implanted with a medical polyethylene pipe (inner diameter 0.28 mm and outer diameter 0.64 mm) at the local site of SCI. The medical polyethylene pipe was connected to a micropump (output efficiency of 1µl/h) embedded under the skin for 3 days.

The present study was approved by the Animal Ethics Committee of the First Affiliated Hospital of Harbin Medical University.

4.2. Basso, Beattie and Bresnahan (BBB) scoring

Behavioral scoring was carried out at different time points after SCI using the BBB scoring system (Thuret et al. 2006). All behavioral observations were performed at the same time (8:00 p.m.) to avoid variations in the movement of animals between day and night.

4.3. Inclined plane test

Behavior was evaluated days 1, 3, 7, 14, 21, and 28 days postoperatively using the modified Rivlin's method (Rivlin and Tator 1977). A simple device was constructed containing a moveable plate with an adjustable angle of 30˚. The rat's head was placed faced forward, and the angle of inclination between the inclined plane and the horizontal plane was increased gradually, until the rats were unable to maintain a constant position (30˚) for 5 s. The angle was considered to be the critical value and then recorded.

4.4. Western blotting

The spinal cords were then processed into homogenized tissue using a homogenizer and then the homogenates were centrifuged at 10,000 × g for 15 min at 4 ˚C. Following this, spinal cord tissues were treated with RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) containing 1% (v/v) phenylmethylsulfonyl fluoride (Beijing Solarbio Science & Technology Co., Ltd.), 0.3% (v/v) protease inhibitor (Sigma-Aldrich; Merck KGaA) and 0.1% (v/v) phosphorylated proteinase inhibitor (Sigma-Aldrich; Merck KGaA). A BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. Subsequently, supernatants were extracted from the lysates following centrifugation at 11,000 x g at 4 ˚C for 15 min. Equal amounts of protein (15 µg/lane) were separated using 10% SDS-PAGE at 300 mA for 2 h and transferred onto a polyvinylidene fluoride membrane. Nonspecific binding was blocked using 8% (w/v) milk in Tris-buffered saline with Tween 20 (TBS-T) for 2 h at room temperature. The following primary antibodies were used: β-actin, cleaved-caspase-3, GRP78, CHOP, caspase-12, ATF6, IRE1αand PERK (all 1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA). Following several washes with Tris-buffered saline with Tween 20, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; ZB-2306, Zhongshan Gold Bridge Biological Technology Co., Beijing, China) for 2 h at room temperature and then washed with TBS-T. Proteins were detected using enhanced chemiluminescence RapidStep™ ECL, according to the manufacturer's protocol (cat. no. 345818, Merck KGaA). ImageJ 1.8.0 (National Institutes of Health, Bethesda, MD, USA) was applied to quantify the relative protein levels. GAPDH was used as an internal control.

4.5. Terminal deoxynucleotidyl-transferase-mediated dUTP nick end (TUNEL)

In the SCI model group, apoptotic cells in the spinal cord were examined after 7 days of SCI, while the number of apoptotic cells in the cAMP group were evaluated 7 days after administration of cAMP. Nuclear fragmentation was detected using TUNEL staining with an In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN, USA) according to the supplier's instructions.

4.6. Statistical analysis

Data were expressed as the mean±standard error of the mean, as indicated. Each experiment was performed in triplicate. Multiple comparisons were performed using one-way analysis of variance followed by Tukey's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference. The data were analyzed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA).

Conflicts of interest: None declared.

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