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ITGB1 suppresses autophagy through inhibiting the mTORC2/AKT signaling pathway in H9C2 cells

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Cardiomyocyte autophagy is closely related to myocardial infarction and hypertrophy. To study the molecular mechanism of autophagy is helpful for the prevention and treatment of these diseases. As a cell surface receptor, the function of ITGB1 gene in cardiomyocyte autophagy is not clear. The purpose of this research was to investigate the function and molecular mechanism of ITGB1 on autophagy. The autophagy-related marker proteins and signaling molecules were detected using western blot with knockdown and overexpression of ITGB1 in H9C2 cells. The results suggested that ITGB1 could inhibit autophagy and the mTORC2/Akt pathway molecules. To further investigate whether the effect of ITGB1 on autophagy might affect myocardial hypertrophy, we constructed Ang II induced H9C2 cells and TAC induced rats models. The results showed that ITGB1 inhibited myocardial hypertrophy in both H9C2 cells and heart tissues of disease model. These data highlight the regulation mechanism on autophagy by ITGB1 and the potential usefulness of the gene as a potential target for preventing heart disease.

1. Introduction

Autophagy is a cyclic process in which cytoplasmic proteins or organelles are phagocytosed and degraded by lysosomes (Wang et al. 2018). It is an important mechanism for maintaining cellular homeostasis. This process is mainly divided into three stages. Firstly, the formation of phagocyte with double membrane structure. Then phosphatidylinositol 3-kinase complexes and ULK complex are recruited to participate in phagocyte formation. Finally, Atg16L complex and Atg8 (LC3) are recruited to participate in phagocyte extension (Levine and Kroemer 2019; Tanida 2011). The dysfunction of cardiac autophagy is closely related to heart failure, cardiac hypertrophy and senescence (Linton et al. 2015; Saito et al. 2016; Schlossarek et al. 2011). Studies have shown that appropriate autophagy can inhibit the progression of cardiac hypertrophy, but excessive autophagy is often associated with adverse reactions such as cardiac hypertrophy and ventricular remodeling (Shires and Gustafsson 2015; Vacek et al. 2012). Thus, further study for the molecular mechanism of cardiomyocyte autophagy is needed for the treatment of heart diseases.

As a key member of integrins family, ITGB1 (Integrin beta1) plays a variety of roles in the cardiovascular system (Henning et al. 2021; Li et al. 2017). Its main function is to mediate cell adhesion and the transformation of intracellular and extracellular signals, which is important for cell communication between intracellular and extracellular information (Gibbs et al. 2018). In recent years, integrin-targeted drugs have shown clear therapeutic effects in the treatment of cardiovascular disease, cancer, inflammatory bowel disease, fibrosis and dry eye disease (Slack et al. 2022). The binding site of extracellular ligand and endogenous ligand can induce intracellular signal transduction by activating or inhibiting the integrin complex and then affecting the level of affinity for extracellular ligand (Slack et al. 2022).

Recent studies show that the LIR motif in integrin β3 can bind to the ATG8 domain of autophagy receptor MAP1LC3 and GABARAP, and is enhanced by the phosphorylation of LIR. This mechanism is involved in coronavirus (sarS-COV-2) reproduction in vivo (Kliche et al. 2021). However, the relationship between ITGB1 and autophagy has not been clearly studied until now. Therefore, this study focuses on the role of ITGB1 for cardiomyocyte autophagy. In our study, we interpreted that ITGB1 suppressed cardiomyocyte autophagy. We utilized rat cardiomyocytes (H9C2 cell line) to explore its possible mechanism and found that mTORC2/Akt pathway could be regulated by ITGB1. In addition, ITGB1 was associated with hypertrophy markers. These results revealed that ITGB1 was a new regulator of autophagy and might be used as a new target molecule for the treatment of heart disease.

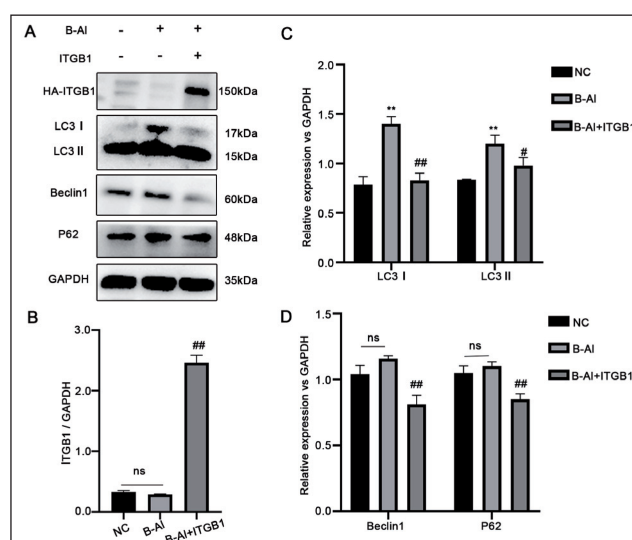

Fig. 1: ITGB1 inhibited autophagy in H9C2 cells incubating with Bafilomycin A1. (A) H9C2 cells were transfected by pcmv-vector or HA-ITGB1 plasmid with or without Bafilomycin A1. Autophagy-related markers, including LC3, Beclin1 and P62 were detected by western blot using their antibodies. (B) Relative expression analysis of ITGB1 for the western blot. (C) Relative expression analysis of LC3 for the western blot. (D) Relative expression analysis of Beclin1 and P62 for the western blot. #: $P<0.05$, ## or **: $P<0.01$.

2. Investigations and results

2.1. *ITGB1 inhibited autophagy in H9C2 cells incubating with Bafilomycin A1*

Since autophagy is a dynamic equilibrium process *in vivo*, we used Bafilomycin A1 to maintain the LC3 protein level. Western blot was used to detect the changes of LC3, Beclin1 and P62 in H9C2 cells with overexpression of ITGB1 (Fig. 1A). The results indicated that Bafilomycin A1 effectively prevented LC3 degradation. In addition, LC3 I was decreased dramatically after ITGB1 transfection (P<0.01), while LC3 II was not significantly changed (Fig. 1B and C). Beclin1, p62 expression were also decreased with ITGB1 transfection in H9C2 cells (P<0.01) (Fig. 1D). Therefore, ITGB1 inhibited autophagy in H9C2 cells incubated with Bafilomycin A1.

2.2. *ITGB1 suppressed cardiomyocyte autophagy in both overexpression and knockdown experiments*

To further clarify the relationship between ITGB1 and autophagy, ITGB1 was overexpressed without the addition of Bafilomycin A1 in H9C2 cells. The results showed that LC3 I and LC3 II expressions were decreased with ITGB1 overexpression compared with the control group (P<0.05) (Fig. 2A and B). To study the effect of endogenous ITGB1 gene on cardiomyocyte autophagy, we prepared small interfering RNA of ITGB1 to silent ITGB1 expression. As shown in Fig. 2B and C, all the three siRNA sequences could effectively disturb ITGB1 expression (P<0.05) (Fig. 2C and D). In addition, ITGB1 knockdown inhibited LC3 I and LC3 II protein level (P<0.05). Therefore, both the overexpression and knockdown results showed that ITGB1 suppressed cardiomyocyte autophagy.

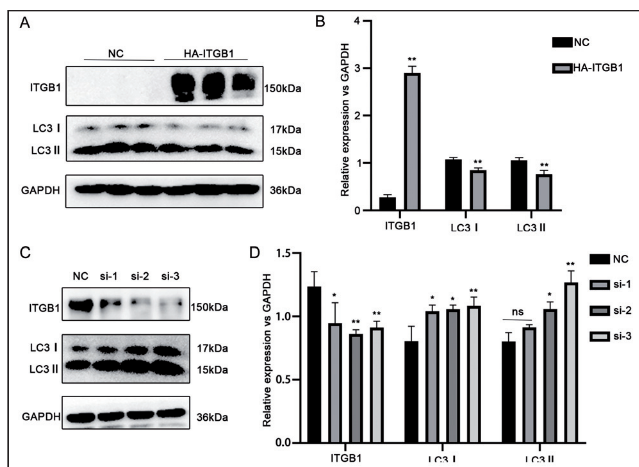

Fig. 2: ITGB1 suppressed cardiomyocyte autophagy in both overexpression and knockdown experiments. (A) H9C2 cells were transfected with pcmv-vector and HA-ITGB1. Autophagy-related marker LC3 was detected by western blot using the antibodies. (B) Relative expression analysis of ITGB1 and LC3 for the western blot. (C) H9C2 cells were transfected by siRNA of ITGB1. Endogenous ITGB1 and LC3 were detected by western blot using their antibodies. (D) Relative expression analysis of ITGB1 and LC3 for the western blot. *: P <0.05, **: P<0.01.

2.3. *ITGB1 inhibited the mTOR/Akt pathway in H9C2 cells*

We then investigated the underlying molecular pathways associated with ITGB1 in regulating cardiomyocyte autophagy, and mTOR pathway was detected. When ITGB1 was overexpressed, phosphorylated mTOR was decreased compared with the control group (P<0.05) (Fig. 3A and B). Studies reported that the regulation of mTOR by ITGB1depended on mTORC2 rather than mTORC1 (Dey-Guha et al. 2015). So the change of mTOR in the western blot results might be induced by mTORC2. To further explore the inhibitory effect of ITGB1 on mTORC2, Akt phosphorylation (Ser473), as the downstream of mTORC2, and its total protein level were detected. Akt phosphorylation (Ser473) was significantly inhibited by ITGB1 with no difference of total protein level (Fig. 3A and B). These results interpreted that ITGB1 inhibited mTORC2 /Akt signaling pathway.

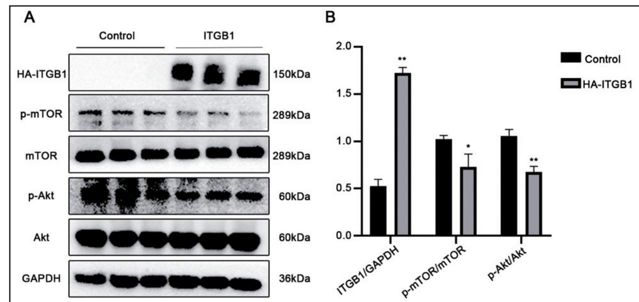

Fig. 3: ITGB1 inhibited the mTOR/Akt pathway in H9C2 cells. (A) H9C2 cells were transfected with pcmv-vector and HA-ITGB1. mTOR signaling pathway related genes, including mTOR, p-mTOR, Akt and p-Akt were detected by western blot using the antibodies. (B) Relative expression analysis of ITGB1, p-mTOR and p-Akt for the western blot. *: P <0.05, **: P<0.01.

2.4. *ITGB1 inhibited Ang II induced hypertrophy markers in H9C2 cells*

In order to further apply the relationship between ITGB1 and heart disease, we stimulated H9C2 cells by Ang II, which could induce hypertrophy of myocardial cells. Western blot detected the expression levels of ANP, BNP and β-MHC with or without ITGB1 overexpression (Fig. 4A and B). The results showed that compared with the control group, the expression of myocardial hypertrophy markers ANP, BNP and β-MHC in Ang II group were significantly increased (P<0.01). Compared with the Ang II group, the expression of ANP, BNP and β-MHC in Ang II + ITGB1 group was significantly decreased (P<0.01) (Fig. 4A and C). The results suggest that ITGB1 could inhibit the hypertrophy markers induced by Ang II in H9C2 cells.

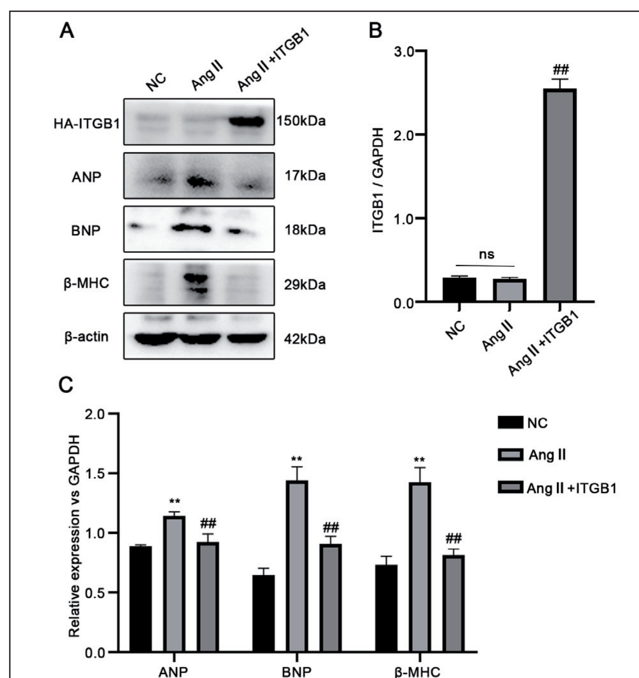

Fig. 4: ITGB1 inhibited Ang II induced hypertrophy markers in H9C2 cells. (A) H9C2 cells were transfected by pcmv-vector or HA-ITGB1 plasmid with or without Ang II. Hypertrophy-related markers, including ANP, BNP and β-MHC were detected by western blot using their antibodies. (B) Relative expression analysis of ITGB1 for the western blot. (C) Relative expression analysis of ANP, BNP and β-MHC for the western blot. ## or **: P<0.01.

2.5. *ITGB1 inhibits autophagy and hypertrophy in TAC model*

To further clarify the role of ITGB1 for autophagy and hypertrophy *in vivo*, we constructed TAC model to induce cardiac hypertrophy in rats. The results of HE staining showed that compared with the sham group, the myocardial tissue arrangement of TAC rats was abnormal and disorder (Fig. 5A). Masson staining showed the excessive deposition of collagen in the interstitium and around blood vessels for the TAC group (Fig. 5B). WGA staining showed the cross-sectional area of single cardiomyocyte cell was increased, which indicated the occurrence of myocardial hypertrophy (Fig. 5C). These results showed that the TAC model in rats was successfully constructed. Immunohistochemical staining of ITGB1 interpreted that ITGB1 expression decreased on the cell surface and around blood vessels in the TAC group compared with the Sham group (Fig. 5D). The decreases of ITGB1 led to increased expression of autophagy markers in TAC-induced hypertrophy animal model, which was consistent with our hypothesis that ITGB1 inhibited autophagy and hypertrophy (Fig. 5E and F).

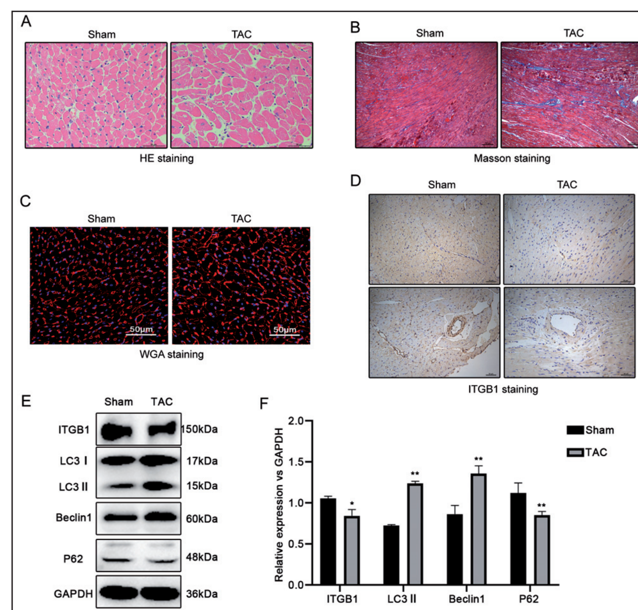

Fig. 5: ITGB1 inhibits autophagy and hypertrophy in TAC model. (A) The cardiac tissue HE staining of control and TAC model rats was performed to detect morphological changes. (B) Masson trichrome staining was analyzed to detect cardiac collagen deposition. (C) WGA staining was performed to detect the cross-sectional area of single cardiomyocyte cell. (D) Immunohistochemistry staining was performed with ITGB1 antibodies in cardiac tissue of control and TAC model rats. (E) ITGB1 protein and autophagy-related markers, including LC3, Beclin1 and P62, were detected by western blot in cardiac tissue lysates of sham-operated and TAC model rats. (F) Relative expression analysis of ITGB1, LC3, Beclin1 and P62 for the western blot. *: $P<0.05$, **: $P<0.01$.

3. Discussion

The importance of the mTOR pathway in autophagy regulation has been demonstrated (Kim and Guan 2015). Two different complexes, mTORC1 and mTORC2, regulate different molecular pathways leading to different cellular responses. Activation of mTORC1 inhibits autophagy, while the role of mTORC2 on autophagy remains controversial (Ballesteros-Alvarez and Andersen 2021; Saxton and Sabatini 2017). Heimbucher's study explored that mTORC2 not only inhibited autophagy through the SGK1 protein, but also promoted autophagy through upmodulating VDAC-1 expression. This finding showed that mTORC2 induced autophagy (Heimbucher et al. 2020). In addition, the regulation of mTOR by ITGB1 depended on mTORC2 rather than mTORC1 (Dey-Guha et al. 2015; Sarbassov et al. 2005). In our study, it was found that autophagy was inhibited with overexpression of ITGB1, and the activity of mTOR was also inhibited. It was reported that Akt (Ser473) was phosphorylated by mTORC2 kinase complex specifically (Ballesteros-Alvarez and Andersen 2021). We further assayed Akt (ser473) with ITGB1 transfection. The reduced phosphorylation of Akt (Ser473) with ITGB1 transfection further confirmed that ITGB1 could inhibited mTORC2 activity (Sarbassov et al. 2005).

The mechanism of pathological hypertrophy of myocardium is still unclear (Shimizu and Minamino 2016). The autophagy activity of cardiomyocytes is closely related to the morphology and function of cardiomyocytes (Bansal et al. 2018). Autophagy plays a dual role in myocardial hypertrophy. Proper autophagy helps to maintain the balance and renewal of the internal environment of the heart, and will not cause excessive damage to cardiac myocytes. However, under certain pathological stimuli, excessive autophagy will occur in cardiac myocytes (Nakai et al. 2007). Excessive autophagy is undoubtedly a harmful manifestation of the heart, and is also an important cause of cardiac hypertrophy (Lavandero et al. 2013). ROS has been found to accumulate significantly during ischemia-reperfusion, leading to progressive myocardial injury. The increase of autophagosome formation during reperfusion is accompanied by significant up-regulation of Beclin1 protein (Matsui et al. 2007). It has been confirmed that autophagy of cardiomyocytes is significantly increased in Ang II or phenylephrine induced cells and TAC induced hypertrophy model (Li et al. 2018). However, in saturated fatty acid induced cardiac hypertrophy, myocardial hypertrophy can be prevented by knockout of LC3B (Russo et al. 2012). In this study, we found that LC3 II and Beclin1 were upregulated and P62 was significantly downregulated in the TAC-induced rats hypertrophy model, indicating that pressure overload led to excessive autophagy of cardiomyocytes, leading to myocardial hypertrophy. In cell experiments, ITGB1 has been confirmed to inhibit autophagy and myocardial hypertrophy. Through *in vitro* and *in vivo* studies, we found that ITGB1 may inhibit cardiac hypertrophy diseases caused by excessive autophagy.

The relationship between ITGB1 and myocardial hypertrophy has also been discussed. In adrenergic stimulated NRVM cells, overexpression of ITGB1 can induce the rise of myocardial hypertrophy marker ANF. But it does not change DNA synthesis (Ross et al. 1998). In our study, overexpression of ITGB1 could inhibit the expression of myocardial hypertrophy markers ANP, BNP and β-MHC in H9C2 cells stimulated by Ang II. So the regulation for heart diseases by ITGB1 was related to the different cell phenotypes and stimulation methods. This study discussed that ITGB1 could inhibit cardiomyocyte autophagy. Maybe some autophagy drugs can be further designed through targeting ITGB1, Our studies provide research basis for related diseases affected by cardiomyocyte autophagy, such as cardiac hypertrophy.

4. Experimental

4.1. *Animals*

Thirty male Sprague-Dawley rats (150-170 g) were purchased from Beijing (China) Charles River Laboratories. All rats were kept under standard temperature 23 °C and humidity (55-60%) with free food and water for 7 days before the experiment. TAC model rats received 1% pentobarbital sodium (0.15 ml/100 g) *via* intraperitoneal injection and awareness was monitored by the negative tail pinch reflex. Then subjected to TAC surgery comprising ligation of the left carotid arteries with a 6–0 silk suture and the sham group received the same procedure but without ligation. There were 10 rats in each group. After feeding for 8 weeks (weight/20+10g), rats were sacrificed by intra-peritoneal injection of pentobarbital sodium, and their heart tissue was collected for further analysis.

4.2. *Cell culture*

The H9C2 cells were obtained from the Procell Life Science&Technology Co., Ltd (Wuhan, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic at 37 °C in a humidified incubator (5% CO_2). DMEM, FBS, 0.25% Trypsin-EDTA, and antibiotic-antimycotic were obtained from Gibco (Grand Island, NY, USA).

4.3. *ITGB1 siRNA transfection*

The ITGB1 siRNA sequences were purchased (GenePharma, Shanghai, China). Lipofectamine 3000 (Thermo Fisher Scientific) was used as transfection reagent.

For reverse transfection, ITGB1 siRNA mixed with transfection reagent was added when cells were plated, followed by the incubation for 68 h at 37 °C in 5% CO_2. The sequences used are shown in the Table.

Table: siRNA sequence for ITGB1

siRNA	sequence
siRNA 1-sense (3'-5')	GCACCAGCCCAUUUAGCUATT
siRNA 1-antisense (5'-3')	UAGCUAAAUGGGCUGGUGCTT
siRNA 2-sense (3'-5')	CCAGACGGAGUAACAAUAATT
siRNA 2-antisense (5'-3')	UUAUUGUUACUCCGUCUGGTT
siRNA 3-sense (3'-5')	CCAAAUAAGGAGUCUGAAATT
siRNA 3-antisense (5'-3')	UUUCAGACUCCUUAUUUGGTT

4.4. HE and masson staining

The ventricles were collected and divided into three parts for the control and TAC models. Firstly, the right ventricle was removed and the left ventricle (LV) was fixed with 4% paraformaldehyde for pathologic analysis. The fixed tissues were subjected to paraffin and cut into 5 μm thick sections. Cross-sections were stained by HE and masson trichrome staining following a method described elsewhere (Li et al. 2021; Sun et al. 2021). The photographs were taken under a light microscope (Leica, Germany and Tissue faxs plus, China). The other parts were stored at −80 °C.

4.5. Immunohistochemistry

The tissue slides (4 μm) were de-paraffinized to water. The epitopes were hot-repaired with sodium citrate buffer (PH=6.0) and naturally cooled to room temperature. After blocking endogenous peroxidase, the cells were sealed with 5% goat serum and incubated at room temperature for 60 min. The primary antibody ITGB1 (Abcam; ab179471, 1:100) was then incubated overnight in 4 °C in a wet box. The next day, prepared DAB chromogenic solution (1:50) was dropped onto the tissue sections for observation under the microscope. Then, hematoxylin restained the nucleus, acidified, dehydrated, transparent, and sealed.

4.6. WGA staining

Paraffin sections were dewaxed conventionally. The antigen epitopes were repaired and cooled to room temperature. Then WGA staining was combined with 1.0 mg/mL WGA stock solution, and incubated at room temperature under dark for 30 min and DAPI for 5 min. Finally, the slides were sealed by anti-fluorescence attenuating tablet and photographed by fluorescence microscope.

4.7. Western blotting

Proteins were incubated with antibody of ITGB1 (Abcam; ab179471, 1:2000), ANP (Abcam; ab225844; 1:1000), BNP (Abcam; ab19645; 1:800), β-MHC (Abcam; ab170867; 1:1000), Akt ser473 (CST; 4691; 1:1000), p-AKT (CST; 9271; 1:1000), mTOR (CST; 2983; 1:1000), p-mTOR Ser2448 (CST; 2983; 1:2971), LC3 (sigma; I7543; 1:1000), Beclin1(CST; 3495; 1:1000), p62 (Bioss; Chain; bs-55207; 1:2000), GAPDH (Proteintech; 60004-1; 1:10000), Beta-Actin (Proteintech; 20536-1-AP; 1:5000) incubated overnight at 4 °C. In the next day the proteins incubated with secondary antibody at room temperature for 1h. Immunoreactive bands were detected by ECL Substrate and visualized by the chemiluminescence imager Omegalum W (the US).

4.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 software (San Diego, CA) and the averaged values were shown as mean±standard deviation (SD). One-way and two-way analysis of variance (ANOVA) were used to determine the statistical significance among groups. Multiple comparison testing was made by the bonferroni multiple comparison test. $P < 0.05$ was considered statistically significant.

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Conflicts of interest: The authors declare no conflict of interest.

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