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microRNA-340 induces apoptosis by downregulation of BAG3 in ovarian cancer SKOV3 cells

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Aberrant expression of miR-340 has been found in several kinds of cancers including ovarian cancer. Pro-apoptotic and anti-metastasis roles of miR-340 in ovarian cancer have also been reported; however, the underling molecular mechanisms by which miR-340 suppresses ovarian cancer are still unclear. This study focused on the role and molecular mechanism of miR-340 in ovarian cancer. Human ovarian carcinoma SKOV3 cells were used and transfected with miR-340 mimic, miR-340 inhibitor and their correspondingly negative controls (mimic control and inhibitor control). Thereafter, cell viability, apoptosis, and the expressions of apoptosis-associated factors and BAG3 were respectively assessed by MTT assay, flow cytometry, qRT-PCR and Western blotting. SKOV3 cells were then co-transfected with miR-340 inhibitor and BAG3 targeted siRNA, then cell viability, apoptosis and the expression of apoptosis-associated factors were retested. Besides, the expressions of main factors in PI3K/AKT pathway were detected. Overexpression of miR-340 suppressed BAG3 cells viability ($P < 0.05$), but improved apoptosis ($P < 0.001$). BAG3 was negatively regulated by miR-340 ($P < 0.05$ or $P < 0.01$). BAG3 silence significantly induced cell apoptosis ($P < 0.001$), and abolished miR-340 suppression-induced increase in cell viability ($P < 0.001$). Besides, BAG3 silence abolished miR-340 suppression-induced activation of PI3K and AKT. This study revealed the tumor suppressive role of miR-340 in SKOV3 cells by negative regulation of BAG3. PI3K/AKT pathway might be involved in the regulation of miR-340 and BAG3.

1. Introduction

Ovarian cancer is one of the most frequent fatal malignancies in middle-aged and elderly females, and is the foremost cause of gynecological cancer death (Koshiyama et al. 2017; Kuzmanov et al. 2013). In recent decades the prognosis of ovarian cancer has been significantly improved, standard therapy includes surgery and chemotherapy resulting in complete clinical remission in up to 75% of patients (Frey and Pothuri 2017; Yi et al. 2017). However, only 30% of women with ovarian cancer will be cured (Frey and Pothuri 2017), and it will recur in more than 50% of patients within 18-24 months. Detailed understanding of ovarian cancer will help to widen new therapeutic strategies of this disease.

microRNAs (miRNAs) are non-coding RNAs with approximately 22 nucleotides, that tune gene expression and modulate target mRNA functions (Pan et al. 2016; Zhu et al. 2016). Recent studies have already reported the importance of miRNAs in a growing number of cancers (Yong and Dutta 2009). In ovarian cancer, some miRNAs have been found abnormal expressed, like Let-7, miR-200 family, miR-17-92, miR-21 (Retamales-Ortega et al. 2017), miR-939 (Ying et al. 2015), and miR-222 (Sun et al. 2013). Some of them have been revealed as oncogenes or tumor suppressors in ovarian cancer. For instance, high expression of the miR-200 family significantly improves overall survival for Asian women with ovarian cancer (Shi and Zhang 2016). Contrary to miR-200, miR-939 was reported functioned as a potential tumor promoter, as evidenced by significant increase in ovarian cancer cells proliferation (Ying et al. 2015).

Aberrant expression of miR-340 has been found in several kinds of cancers, including non-small cell lung cancer (Fernandez et al. 2015), breast cancer (Chen et al. 2015), gastric cancer (Hou and Qiao 2015), and ovarian cancer (Li et al. 2016), indicating that miR-340 might play a critical role in tumorigenesis. Moreover, it exerted anti-growth, pro-apoptotic, and anti-metastasis activities in these cancers via regulation of its target gene like p27, MYO10, and NF-x03BA;B1 (Chen et al. 2015; Fernandez et al. 2015; Li et al. 2016), revealing the tumor suppressive role of miR-340 in cancers.

Despite the pro-apoptotic and anti-metastasis roles of miR-340 in ovarian cancer have been reported by Li et al. (2016), the underling molecular mechanisms via which miR-340 suppresses ovarian cancer are still unclear. Thus, this study by focused on the role and molecular mechanism of miR-340 in ovarian cancer. We used human ovarian carcinoma SKOV3 cells, and the expression of miR-340 in this cell line was overexpressed by transfection with miR-340 mimic, while was suppressed by transfection with miR-340 inhibitor. Thereafter, cell viability, apoptotic cells rate and the expression of apoptosis-associated factors were assessed. Furthermore, the regulation of miR-340a and BAG3 were detected to reveal whether BAG3 was involved in miR-340-mediated SKOV3 cells.

2. Investigations and results

2.1. miR-340 overexpression suppressed SKOV3 cells viability but induced apoptosis

SKOV3 cells were transfected with miR-340 mimic, miR-340 inhibitor or their correspondingly negative controls (mimic control and inhibitor control), and then transfection efficiency was verified by detection the expression of miR-340. Results from qRT-PCR (Fig. 1A), showed that the level of miR-340 was increased after miR-340 mimic transfection ($P < 0.001$), while was reduced after miR-340 inhibitor transfection ($P < 0.01$), when compared with

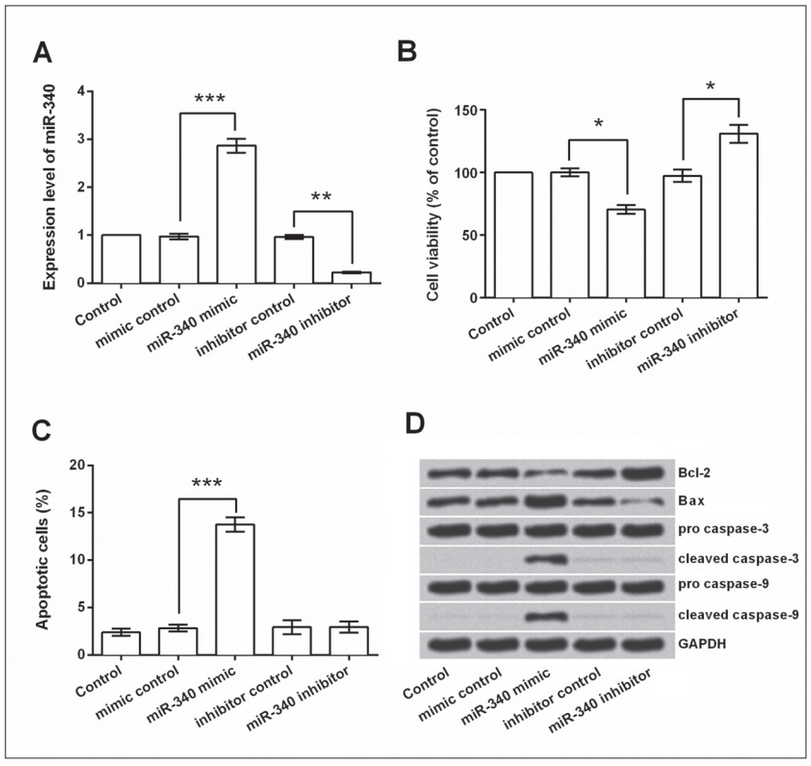

Fig. 1: miR-340 overexpression suppressed SKOV3 cells viability but induced apoptosis. SKOV3 cells were transfected with miR-340 mimic, miR-340 inhibitor or their correspondingly negative controls (mimic control and inhibitor control). (A) Transfection efficiency was verified by qRT-PCR. (B) Cell viability (C) apoptotic cells rate, and (D) the expressions of apoptosis-associated proteins were respectively assessed by MTT, flow cytometry and Western blotting. n = 3. Data were expressed as mean±SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (ANOVA).

their correspondingly controls. Results from MTT assay (Fig. 1B) showed that, cell viability was much lower in miR-340 overexpressing-cells, while was higher in miR-340 suppressing-cells (both $P < 0.05$) compared to their correspondingly controls. Flow cytometry detection results given in Fig. 1C showed that, apoptotic cells rate were increased by miR-340 overexpression ($P < 0.001$), while miR-340 suppression had no significant impact on apoptotic cells rate. In addition, Western blot was performed and as results shown in Fig. 1D, down-regulation of Bcl-2, upregulation of Bax, as well as activation of caspase-3 and capsase-9 were found in miR-340 overexpressing-cells. In contrast, upregulation of Bcl-2, and down-regulation of Bax were found in miR-340 suppressing-cells. No impact of caspase-3 and caspase-9 in miR-340 suppressing-cells was observed. These data revealed the tumor suppressive effects of miR-340 on SKOV3 cells.

2.2. BAG3 was negatively regulated by miR-340

Given that BAG3 is constitutively expressed in several primary tumours or tumour cell lines, and it has been shown to exert pro-survival and anti-apoptotic roles through various mechanisms (Kong et al. 2016; Rosati et al. 2015). We asked whether miR-340 could alter the expression of BAG3 in this study. As results shown in Fig. 2A and 2B, both the mRNA and protein level expressions of BAG3 were downregulated after miR-340 was overexpressed in SKOV3 cells ($P < 0.05$), while were upregulated after miR-340 was suppressed ($P < 0.01$). These data suggested that BAG3 was negatively regulated by miR-340 in SKOV3 cells.

2.3. miR-340 suppressed SKOV3 cells via regulation of BAG3

Next, miR-340 inhibitor and BAG3 siRNA were co-transfected into SKOV3 cells, to explore whether BAG3 was implicated in the tumor suppressive effects of miR-340. As results shown in Fig. 3A and 3B, BAG3 silencing largely reduced cell viability even if miR-340 was suppressed in cells ($P < 0.001$), and dramatically increased apoptotic cells rate ($P < 0.001$). Further, western blotting analytical results (Fig. 3C) showed that, BAG3 silencing

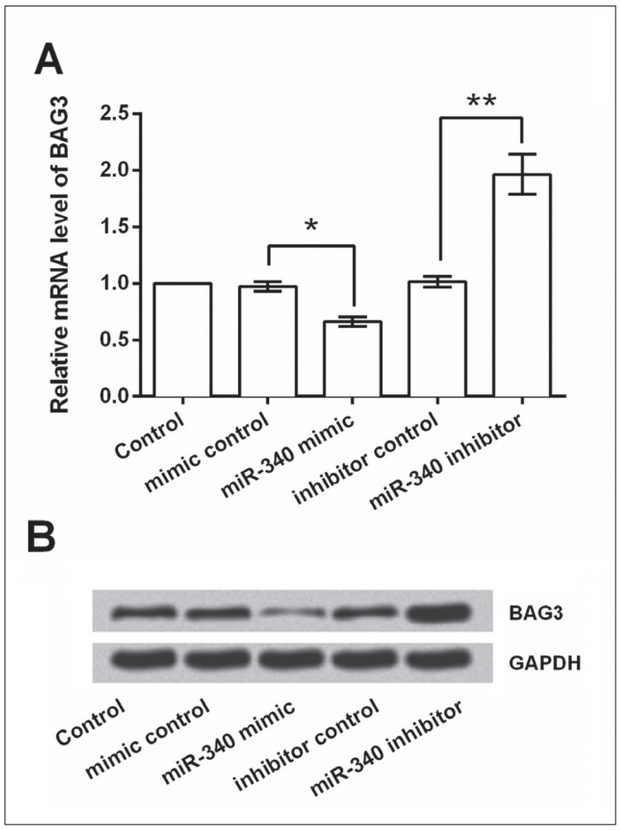

Fig. 2: BAG3 was negatively regulated by miR-340. SKOV3 cells were transfected with miR-340 mimic, miR-340 inhibitor or their correspondingly negative controls (mimic control and inhibitor control). The (A) mRNA and (B) protein level expressions of BAG3 were respectively detected by qRT-PCR and Western blot analyses. n = 3. Data were expressed as means ± SD. * $P < 0.05$, ** $P < 0.01$ (ANOVA).

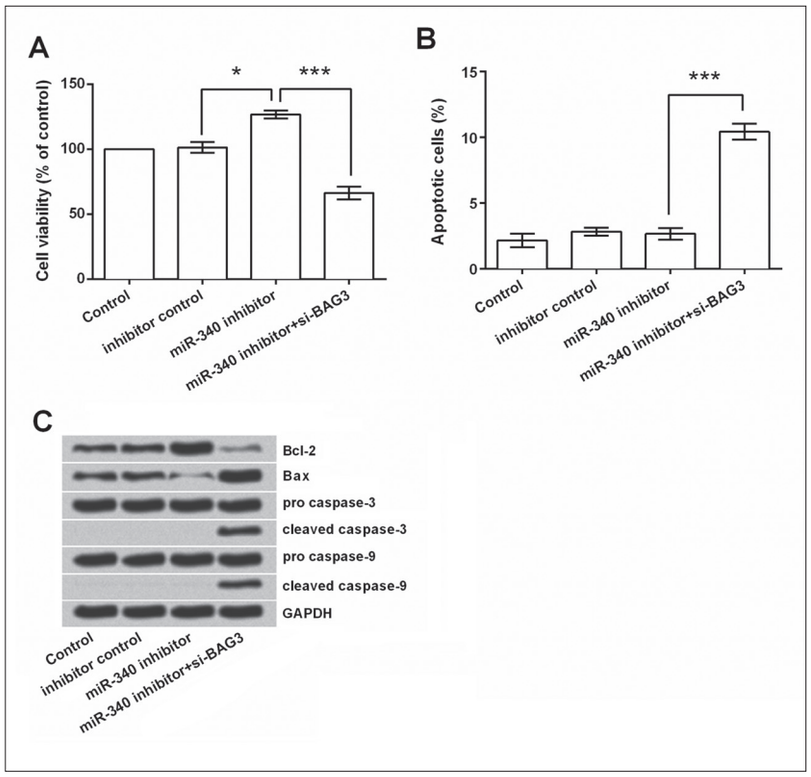

Fig. 3: miR-340 suppressed SKOV3 cells via regulation of BAG3. SKOV3 cells were co-transfected with miR-340 inhibitor and BAG3 targeted siRNA. (A) Cell viability (B) apoptotic cells rate, and (C) the expressions of apoptosis-associated proteins were respectively assessed by MTT, flow cytometry and Western blotting. n = 3. Data were expressed as means ± SD. * $P < 0.05$, *** $P < 0.001$ (ANOVA).

remarkably recovered miR-340-induced abnormal expressions of apoptosis-associated proteins, as evidenced by the downregulation of Bcl-2, the upregulation of Bax, and activations of caspase-3 and caspase-9. These data suggested miR-340 suppressed SKOV3 cells viability and improved apoptosis possibly via negative regulation of BAG3.

2.4. The regulation of miR-340 and BAG3 involved in PI3K/AKT pathway

PI3K/AKT pathway is activated in approximately 70% of ovarian carcinomas (Chen et al. 2016), and activation of this pathway is associated with tumour cells proliferation, apoptosis and metastasis. Thus, we detected the expression of main factors in this pathway to detect whether PI3K/AKT pathway was implicated in miR-340 mediated SKOV3 cells. As shown in Fig. 4, BAG3, p-PI3K, and p-AKT were all upregulated by miR-340 suppression, while were remarkably downregulated after BAG3 was silenced. These data provide evidence that PI3K/AKT pathway might be involved in the regulation of miR-340 and BAG3.

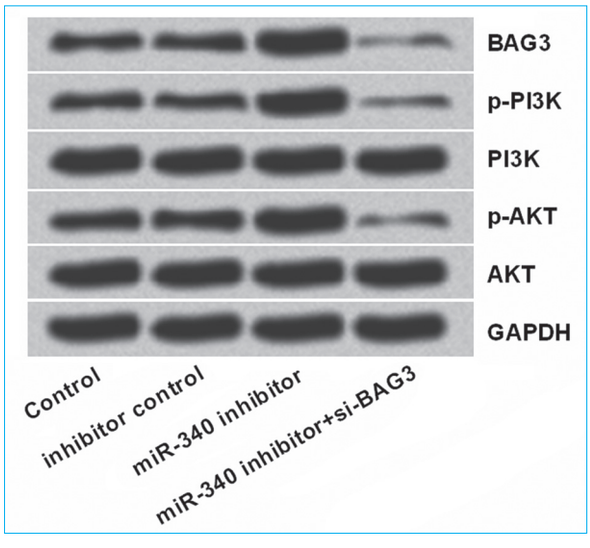

Fig. 4: The regulation of miR-340 and BAG3 involved in PI3K/AKT pathway. SKOV3 cells were co-transfected with miR-340 inhibitor and BAG3 targeted siRNA. The expressions of BAG3, p-PI3K, PI3K, p-AKT and AKT were detected by Western blotting.

3. Discussion

In the current study, we demonstrated that miR-340 overexpression inhibited SKOV3 cells viability but improved apoptosis, indicating miR-340 might serve as tumor suppressor in ovarian cancer. BAG3 silence significantly induced cell apoptosis, and abolished miR-340 suppression-induced increase in cell viability. Besides, BAG3 silence abolished miR-340 suppression-induced activation of PI3K and AKT.

Many reports on the function of miR-340 in human tumorigenesis have been published (Chen et al. 2015; Fernandez et al. 2015; Hou and Qiao 2015; Li et al. 2016). Previous studies have also reported that miR-340 overexpression inhibited ovarian cancer cell lines (CAOV3 and A2780 cells) proliferation and promoted apoptosis (Li et al. 2016). In this study, we confirmed the tumor suppressive roles of miR-340 in another ovarian cancer cell line, *i.e.*, SKOV3 cells, as evidenced by the decrease in cell viability, increase in apoptotic cells rate, downregulation of Bcl-2, upregulation of Bax, and activation of caspase-3 and caspase-9.

BAG3, a member of the BAG family of proteins (BAG1-BAG6), has been identified as co-chaperone and cell survival protein (Liang et al. 2017; Takayama and Reed 2001). BAG3 regulates multiple cellular behaviours, including cell division, migration, differentiation and death (Antonietti et al. 2017; Carrizzo et al. 2016; Takayama and Reed 2001). Besides, BAG3 is constitutively expressed in several tumours, and acts as an oncogene by controlling tumour cells proliferation and apoptosis (Kong et al. 2016; Rosati et al. 2015). In terms of ovarian cancer, BAG3 has been revealed to be associated with a poor prognosis of primary ovarian cancer (Habata et al. 2016). BAG3 positivity was higher at advanced clin-

ical stages of ovarian cancer than at early stages, and it interacted with MMP-2 to positively regulate ovarian cancer cells invasion (Suzuki et al. 2011). In consistence with these previous studies, we confirmed the carcinogenic effects of BAG3 in ovarian cancer, that BAG3 silence inhibited SKOV3 cells viability and induced apoptosis. Besides, we for the first time proposed that miR-340 suppressed SKOV3 cells via negative regulation of BAG3, since BAG3 silence abolished miR-340 suppressing-induced increase in cell viability, and miR-340 suppressing-induced abnormal expressions of apoptosis-associated proteins. However, more efforts are still needed for further confirming this hypothesis.

The PI3K/AKT pathway is activated in approximately 70% of ovarian cancers (Chen et al. 2016), and it contributes to ovarian cancer cells apoptosis, cell cycle, autophagy and epithelial to mesenchymal transition (EMT) processes (Bugide et al. 2016; Zi et al. 2015). PI3K is upstream of AKT and the phosphorylation of AKT can be upregulated following the activation of PI3K. In laryngeal squamous cell carcinoma cells, miR-340 has been found to suppress PI3K/AKT activation (Yu et al. 2016). On the other hand, it has been reported that BAG3 exerted its vasorelaxant effect on resistance vessels by activation of the PI3K/AKT pathway (Carrizzo et al. 2016). However, our study for the first time linked the regulation between miR-340-BAG3 axis and PI3K/AKT pathway, providing evidence that miR-340 negatively regulated BAG3 and thus inactivated PI3K/AKT pathway in SKOV3 cells.

To sum up, we revealed the tumor suppressive role of miR-340 in SKOV3 cells, as miR-340 overexpression inhibited cell viability, and improved apoptosis. BAG3 was negatively regulated by miR-340, and miR-340 suppressed SKOV3 cells possibly via negative regulation of BAG3. Besides, the PI3K/AKT pathway might be involved in the regulation of miR-340 and BAG3. This study will provide a better understanding of miR-340 in ovarian cancer, and suggest its potential application in the treatment of this cancer.

4. Experimental

4.1. Cell culture and transfection

Human ovarian carcinoma SKOV3 cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St Louis, Missouri) in a humidified atmosphere of 5% CO_2 at 37 °C. For cell transfection, miR-340 mimic, miR-340 inhibitor, and their negative controls (mimic control and inhibitor control) were purchased from GenePharma Co. (Shanghai, China). The specific siRNA against BAG3 was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). All transfections were conducted using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. At 48 h of transfection, cells were collected for use in the forthcoming analyses.

4.2. Cell viability assay

Cell viability was determined by using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, the transfected-cells were seeded in 96-well plates at a density of 5×10^3 cells/well for adherence. After 48 h of incubation, 20 μL CCK-8 solution was added and the plates were incubated in 37 °C for another 3 h. The absorbance was measured by using a Microplate Reader (Bio-Rad, Hercules, CA) at 450 nm.

4.3. Apoptosis assay

Apoptotic cells were stained by Annexin V-FITC/PI Kit (4 A Biotech Co. Ltd., Beijing, China) and analyzed by flow cytometric analysis. In brief, 1×10^5 of transfected cells were collected and re-suspended in 200 μL binding buffer containing 10 μL Annexin V-FITC. After a 30 min incubation at room temperature in the dark, 5 μL PI and 300 μL phosphate buffered saline (PBS) were added, and the samples were immediately analyzed on the flow cytometry (Beckman Coulter, Fullerton, CA, USA) (Pan and Zhao 2015).

4.4. qRT-PCR

Total RNA in transfected cells was extracted by Trizol Reagent (Invitrogen, Carlsbad, CA, USA). For miR-340 detection, cDNA was synthesized by using PrimeScript RT reagent Kit (TaKaRa, Dalian, China), and qRT-PCR was performed by using SYBR Premix ExTaq (TaKaRa) according to the manufacturer's instructions. For BAG3 detection, cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). qRT-PCR was performed using FastStart Universal SYBR Green Master (Roche). Each qRT-PCR was carried out in triplicate for a total of 20 μL reaction mixtures on ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Expression of miR-340 was normalized against the endogenous snRNA U6 control, and BAG3 was normalized to GAPDH. Data were analyzed according to the classic $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

4.5. Western blot analysis

Total protein in the transfected cells were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche). The proteins concentration was determined by BCA Protein Assay Kit (Pierce, Appleton, WI, USA). Protein (0.1 mg) from each sample was resolved over 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (Millipore, Bed-ford, MA, USA). After blocking in 5% non-fat milk for 1 h at room temperature, the membranes were incubated with primary antibody at dilution of 1:1,000 for the specific detection of Bcl-2 (ab32124), Bax (ab32503), pro caspase-3 (ab13847), cleaved caspase-3 (ab2302), pro caspase-9 (ab32539), cleaved caspase-9 (ab2324), BAG3 (ab47124), p-PI3K (ab182651), PI3K (ab86714), p-AKT (ab8932), AKT (ab8805), and GAPDH (ab9485, Abcam, Cambridge, MA) at 4°C overnight. The membranes were then incubated with secondary antibodies (1:2,000 dilution, Abcam) for 1 h at room temperature. Positive signals were visualized by enhanced chemiluminescence (ECL) method.

4.6. Statistical analysis

All data are expressed as mean±SD from three independent experiments in triplicate. Statistical differences between groups were performed by GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) by using a one-way analysis of variance (ANOVA). Results with P-value of < 0.05 were considered as statistically significant results.

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Conflict of interests: There is no conflict of interests.

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