Melatonin ameliorates ovarian hyperstimulation syndrome (OHSS) through SESN2 regulated antiapoptosis

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Abstract

Research question: Ovarian hyperstimulation syndrome (OHSS) is one of the most severe complications after ovarian stimulation during assisted reproductive technology (ART). However, its pathogenesis still remains unclear. Melatonin is an important antioxidant factor in female reproduction and Sestrin-2 (SESN2) is reported to be involved in cellular response to different stress conditions. Whether or not melation and SESN2 are involved in OHSS is still a question to us clinicians.

Design: We collected the granulosa cells of OHSS patients and focused on the role of SESN2 in OHSS. We also studied the role and mechanism of melatonin plays in OHSS patients.

Results: We found that the expression of SESN2 was increased in the granulosa cells of OHSS patients (n=24) than those in controls (n=15). Incubation with angiotensin II (1μM, 2μM) in HUVECs and H_2O_2 (0.1mM, 0.2mM) in KGNs increased the generation of ROS concurrent with the increased expression of SESN2, while melatonin treatment partly restored SESN2 levels. Mechanism study demonstrated that SESN2 was deeply involved in the regulation of AMPK and mTOR, whearas melatonin partially restored angiotensin II or H_2O_2 induced the activation of AMPK phosphorylation and the inhibition of mTOR, 4EBP1 and S6K1 phosphorylation, all of which could trigger cell apoptosis. Additionally, we found that SESN2 knockdown and treatment with rapamycin (mTOR inhibitor) in HUVECs partly abolished the antiapoptosis effects of melatonin.

Conclusions: These findings indicated that melatonin attenuated ROS-induced apoptosis through SESN2-AMPK-mTOR in OHSS. Thus, melatonin is likely to be a potential and important therapeutic agent for treating and preventing OHSS.

Introduction

Infertility is defined as the inability to be pregnant within one year of unprotected intercourse and its incidence has reached to 10–15% in recent years.[Maya et al.,2012]. It has become not only a medical concern, but also a social issue with increasing prevalence in both developed and developing countries. In vitro fertilization (IVF) is wildly accepted over the past 35 years as an effective treatment for infertility during which controlled ovarian stimulation (COH) is almost always employed to retrieve more oocytes. Although COH might improve IVF outcome, it also increases the risk of an iatrogenic complication: ovarian hyperstimulation syndrome (OHSS). OHSS is not uncommon. Studies reported that the presence of its moderate and severe form was up to 10% of all IVF cycles [Asch et al.,1991]. The true incidence of OHSS is probably much underestimated since the symptoms of mild OHSS are easy to be ignored [Ryan et al.,2014]. The symptoms of OHSS in its mild form can be untypical such as nausea, vomiting, however, the moderate and severe OHSS may result in oliguria, hydrothorax, ascite, hepatorenal failure, acute respiratory distress syndrome, hemorrhage from ovarian rupture, thromboembolism even ultimately, death [Mocanu et al., 1997]. Although OHSS increases the physical, psychological and economic burden of the patients and their families, its pathogenesis is not completely understood and no specific therapy

is available for this syndrome. Therefore, prevention of OHSS becomes a crucial issue since its treatment is largely tamping down symptoms, rather than addressing causes.

The precise cause of OHSS remains currently the subject of controversy. Nevertheless, high estradiol levels in the presence of human chorionic gonadochotropin (hCG) increase the vascular endothelium permeability, leading to a massive shift of intravascular fluid into the third space. There are also evidences that during the pathogenesis of this iatrogenic complication, large amounts of angiotensin II, vascular endothelial growth factor (VEGF), interleukins (ILs), nitric oxide (NO), tumor necrosis factor-a(TNF-a)and other molecules are excessively produced, causing the overproduction of reactive oxygen species (ROS) which results in oxidant-antioxidant imbalance[McDonough 2003]. The vascular endothelium is then deteriorated by these imbalanced free radicals that cannot be antagonized by free radical scavengers, consequently, high vascular permeability occures and finally results in the aggravation of OHSS [Gomez et al.,2010].

Melatonin is mainly secreted by pineal glands in human beings and is regulated by circadian rhythms. However, higher levels of melatonin are found in human follicular fluid than in plasma because melatonin is not only derived from the general circulation but also synthesized in the ovary (mainly by the granulosa cells) [Brzezinski et al.1987]. Melatonin has a significant impact on female reproduction. It is considered essential for folliculogenesis, steroid production. There are also evidences that melatonin takes part in the control of pubertal onset, ovulation, sexual maturation and pregnancy protection[Hattori et al.,2007]. Melatonin as well as its metabolites has been proved to be a powerful radical scavenger. It reduces ROS levels in the ovary through receptor dependent and independent pathways [Zavodnik et al.,2006; Kang et al.,2009]. Recently, more and more attention has been paid to the importance of melatonin in female reproduction [Tamura et al.,2008].

Sestrins are highly conserved and stress-inducible metabolic regulators which are ubiquitously expressed at different levels in all adult tissues [Velasco-Miguel et al., 1999; Qyerfyrtg et ak,,2010]. The physiological functions of sestrins have not been fully elucidated yet. The critical roles of sestrins in mammalian metabolism have been revealed by the deletion of these proteins which is incompatible with the survive of mice[Peng et al.,2014].Previous studies have also suggested that sestrins have close relationship with age and oxidative stress associated diseases such as Alzeimer’s disease, Parkinson’s disease, diabetes, etc and have a favorable profile as potential therapeutic targets for these diseases[Schapira et al.,2010; Lee et al., 2010]. Sestrin-2 (SESN2) belongs to the sestrins family and functions as a suppressor of ROS accumulation as well as a neuroprotector [Wullschleger et al.,2006]. The overexpression of SESN2 reduces ROS levels whereas SESN2 knockdown in cultured cells or mice increases ROS content[Budanov et al.2001;Kopnin et al.,2001]. Moreover, any condition that leads to ROS accumulation induces SESN2 expression[Lee et al.,1999]. Therefore, the increased ROS levels in OHSS may increase the expression of SESN2. Most previous studies on SESN2 have been focused on nervous system and cardiovascular system, these studies have shown that SESN2 played an important role in preventing ROS damage, repairing mitochondria deterioration and maintaining the stability of inner environment [Zhou et al.,2013; Chen et al.,2017]. However, there are very few studies on the expression and the function of SESN2 in

human reproduction. Since OHSS is closely associated with the excessive production of ROS and melatonin is supposed to be a powerful radical scanvenger, therefore, the objective of this study was to investigate whether SESN2 are induced in OHSS and whether melatonin can alleviate oxidative stress in OHSS as well as the potential role of SESN2 in OHSS.

Materials And Methods

Clinical sample collection

Patients with high serum E_2 levels (≥ 5000 pg/ml) on the day of human chorionic gonadotropin (hCG) administration and ovulated more than 20 oocytes were identified as patients at high risk of OHSS[Papanikolaou et al.,2006]. Cases with relatively low level of E_2 (< 4000 pg/ml) and the number of retrieved oocytes < 20 were chosen as the control group. Clinical parameters including age, BMI, AMH, basal FSH, basal E_2, E_2 on hCG administration day, number of oocytes retrieved were collected. Patients received hCG when the diameter of their follicles was > 18 mm. Oocytes were collected 36 h after hCG injection by transvaginal ultrasound-guided puncture and aspiration of the follicles with a diameter of 18 to 20 mm. The granulosa cells from patients on oocyte retrieval day were collected and purified with Ficoll-Pague™ PLUS (GE-HealthCare Bio-Science, Uppsala, Sweden). Our research was approved by the Reproductive Ethics Committee of Ren Ji Hospital. The informed consent was obtained from each patient before oocyte retrieval.

Cell culture

human umbilical vein endothelial cells (HUVECs, JCRB Cat# IFO50271, RRID:CVCL_2959) and human granulosa cell line (KGNs, RCB Cat# RCB1154, RRID:CVCL_0375) were kindly provided by Shandong University. HUVECs represent endothelial cells and KGNs are used to represent ovarian granulosa cells in previous studies[Gaytan et al.,2018]. The cells were inoculated in 75cm^2 cell culture flasks and cultured in Phenol Red-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco, USA), containing 10% fetal bovine serum (Gibco, USA) and 1% Penicillin-Streptomycin (Gibco, USA). The cells were passaged every 2 days and incubated at 37℃ with 5% CO_2 in a humidified atmosphere. The cells were then digested and counted before being seeded in six-well plates.

The evaluation of intracellular ROS of cultured cells

Intracellular ROS levels were measured using fluoroprobe CM-H_2DCFDA (Sigma #D6883, Sigma-Aldrich). Cells cultured in six-well plates were incubated in DMED/F12 with 10 mM CM-H_2DCFDA for 20 min at 37℃ in a dark, humidified chamber. After incubation, the cells were washed with PBS twice. CM-H_2DCFDA fluorescence was measured using a fluorescence microscope (Zeiss, Germany) with a digital imaging system at an excitation wavelength ranging from 430 to 480 nm and the intensity of the fluorescence were analyzed using IMAGE J 1.34 s (National Institutes of Health, USA).

RNA extraction and real-time PCR

Total RNA from cells was extracted using total RNA isolation kit (Foregene, China) following the manufacturers' instructions and reversely transcripted into cDNA using ReverTra Ace qPCR RT Kit (Toyobo, Japan). Real-time PCR was performed using an Applied Biosystem Real-Time PCR system (Life Technologies Inc.) with the following conditions: initial denaturation for 10 min at 95℃, followed by 40 cycles of 10 s at 95℃, 20 s at 59℃, 20 s at 72℃;and a final extension for 5 min at 72℃. Real-time PCR reactions were performed in triplicate with SYBR GREEN PCR Master Mix (Toyobo, Japan). Melting-curve analyses were carried out to verify the product identities. The expression of target genes were detected by real-time polymerase chain reaction and the results were analyzed by ΔΔCt methods [Li et al.,2017;Li et al.,2018; Wang et al.2020]. ß-actin was chosen as an internal control. The sequences of the primers used for amplification are:

SESN2 5'-TCTTACCTGGTAGGCTCCCAC-3'

5'-AGCAACTTGTTGATCTCGCTG-3'.
ATCB 5'-CTCCATCCTGGCCTCGCTGT-3'

5'-GCTGTCACCTTCACCGTTCC-3.

Western Blot

Total protein was isolated using RIPA buffer (Beyotime Biotechnology, China) with protease inhibitors (Sigma Chemical Co., USA). Isolated proteins were quantified with an Enhanced BCA Protein Assay kit (Thermo Fisher Scientific, USA). 30μg proteins were applied to an SDS gel for electrophoresis and were then transferred to polyvinylidene fluoride membranes. Non-specific binding site were blocked using 5% non-fat milk for 90 min at room temperature. After blocking, the membrane was incubated overnight at 4℃ with the following primary antibodies: anti-SESN2 antibody (1:1000, Proteintech #10795, Proteintech Group Inc, RRID:AB_10658316), anti-p-S6K1^{Thr389} antibody (1:1000, CST #9234, Cell Signaling Technology, RRID:AB_2568667), anti-S6K1 antibody (1:1000, CST #2708, Cell Signaling Technology, RRID:AB_2569704), anti-BCL2 antibody (1:1000, Proteintech #12789, Proteintech Group Inc, RRID:AB_1051486), anti-p-4EBP1$^{Thr37/46}$ antibody (1:1000, CST #2855, Cell Signaling Technology, RRID:AB_2097980), anti-p-mTOR^{Ser2448} antibody (1:1000, CST #5536, Cell Signaling Technology, RRID:AB_2861149), anti-mTOR antibody (1:1000, CST #2983, Cell Signaling Technology, RRID:AB_385472), anti-p-AMPK^{Thr173} antibody (1:1000, CST #2531, Cell Signaling Technology, RRID:AB_1609276), anti-AMPK antibody (1:1000, CST #2603, Cell Signaling Technology, RRID:AB_1553973), anti-p17 caspase 3 antibody (1:1000, Proteintech #25546, Proteintech Group Inc., RRID:AB_2746035), anti-caspase 3 antibody (1:1000, Proteintech #19677, Proteintech Group Inc., RRID:AB_367945), anti-GAPDH antibody (1:1000, Proteintech #60004, Proteintech Group Inc., RRID:AB_1616728). After washed with Tris-buffered saline containing 0.1% Tween-20 three times, the membrane was incubated with diluted peroxidase-conjugated secondary antibodies for 1h at room temperature. The protein signals were developed using ECL chemiluminescence kit (Millipore, USA). The bands were then analyzed using Quantiscan software (Biosoft, UK)[Guo et al.,2015;Meng et al.,2016; Chen et al., 2018; Cui et al.,2011;Guo et al.,2018].

SESN2 small interfering RNA (siRNA) knocking down

SiRNA were purchased from Invitrogen. HUVECs were plated in medium without antibiotics at approximately 4×10^5 cells per well in six-well plates. The mixture of siRNA (2.5μl/per well) and Lipofectamine 2000 Transfection Reagent (9μl/per well; Invitrogen) in OPTI-MEM medium (Gibco, USA) was incubated at room temperature for 5 min. The mixtures were then added to the cells in six-well plates. After 24 h, the medium was removed and the following experiments were continued. The specific sequences of SESN2 siRNA are as follows:

5’-GCUCGGAAUUAAUGUGCCATT-3’ 5’-UGGCACAUUAAUUCCGAGCTT-3’

Statistical analysis

All data were analyzed using SPSS 22.0 software (IBM, USA). The differences between two groups were analyzed using student’s t-test. The differences among three groups or above were analyzed using one-way anova analysis followed by the Newman-Keuls multiple comparison test. The data are represented as mean ± standard deviation (SD) and all experiments were repeated at least three times. $P < 0.05$ was regarded as statistically significant.

Results

Patients at high risk of OHSS showed an increased expression of SESN2 in granulosa cells

The clinical characteristics of the patients are presented in Table 1. The OHSS patients had younger age, lower BMI, lower base FSH levels than the women in non-OHSS group and higher AMH levels, higher E_2 levels on hCG day, moreover, OHSS patients retrieved more oocytes than non-OHSS group ($P < 0.05$). There was no difference of base E_2 levels between the two groups. Furthermore, the suppressor of ROS accumulation, SESN2, both its mRNA (OHSS n = 24, control n = 15) ($p = 0.003$) and protein levels (OHSS n = 24, control n = 15) ($p = 0.038$) were significantly increased in the granulosa cells of high risk OHSS patients (Fig. 1A,B,C).

Table 1
Clinical characteristics of the study population

parameters	control (n = 15)	OHSS (n = 24)	P value
age(years)	30.13 ± 3.54	27.08 ± 3.17	0.025*
BMI(kg/m^2)	22.78 ± 2.83	20.37 ± 2.00	0.017*
AMH(ng/ml)	2.81 ± 1.07	13.34 ± 5.68	0.001**
bFSH(mIU/ml)	7.06 ± 1.23	5.75 ± 1.06	0.006**
bE_2(pg/ml)	40.71 ± 20.02	42.30 ± 18.02	0.828
E_2 on hCG day(pg/ml)	2426.53 ± 1325.46	8589.23 ± 2623.04	0.001**
number of oocytes retrieved(n.)	10.87 ± 5.15	37.92 ± 11.00	0.001**

*P < 0.05 compared with control group; **P < 0.01 compared with control group.

SESN2 levels were in parallel to the ROS generation in HUVECs and KGNs

Since OHSS is highly associated with the excessive production of ROS, which induces the deterioration of endothelial cells and the dilatation of vessels in whole body, we explored SESN2 and ROS levels in OHSS. We chose HUVECs as endothelial cells model and had them cultured with Angiotensin II to build OHSS oxidative stress model. Incubation with angiotensin II (1μM, 2μM) significantly increased the generation of ROS (1μM angiotensin II versus control: 3.53 ± 0.28 versus 1.00, P = 0.034; 2μM angiotensin II versus control: 5.46 ± 0.98 versus 1.00, P = 0.005) (Fig. 2A, B). The SESN2 levels were also significantly increased (P = 0.042) (Fig. 2D, F). Therefore, the SESN2 levels were in parallel to the ROS generation in HUVECs.

To further confirm the results observed, we also used KGNs cells to mimic ovarian granulosa cells. The same phenomenon was observed in KGNs cells. The ROS generation and SESN2 levels in cultured KGNs cells were significantly increased after incubation with H_2O_2 (0.1mM, 0.2mM) (Fig. 2A, C, E, G).

Melatonin significantly restored SESN2 levels in cultured HUVECs and KGNs

Since ROS and SESN2 levels are increased in OHSS model and melatonin is a powerful radical scavenger, we explored the role of melatonin in treating OHSS. The treatment with melatonin (10μM) for 24 h significantly decreased SESN2 level induced by angiotensin II (P = 0.037) (Fig. 3A, B), similarly, the

treatment with melatonin (1mM) for 12h significantly decreased SESN2 level in KGNs cells induced by H_2O_2 (Fig. 3C, D).

Melatonin inhibited apoptosis through SESN2 regulated signaling pathway in HUVECs and KGNs

Previous studies have demonstrated that melatonin plays an antioxidant role and regulates antiapoptosis pathway[Ma et al.,2018; Shi et al.,2018]. To better investigate the role and mechanism melatonin plays in OHSS, we determined the classic apoptosis related molecule levels in angiotensin II induced HUVECs treated with melatonin. The results demonstrated that incubation with angiotensin II (1μM) significantly increased the levels of p17 caspase 3 and decreased BCL2 level, which were partly restored by the treatment with melatonin (10μM)($P < 0.05$) (Fig. 4A,B). SESN2 has previous been demonstrated to be involved in the AMPK-mTOR signaling pathway which is related to apoptosis. Therefore, we studied this signal pathway and found that incubation with angiotensin II (1μM) in cultured HUVECs significantly increased the levels of p-AMPK and decreased the levels of p-mTOR and its downstream molecules(p-S6K1, p-4EBP1), which were partly restored by the treatment with melatonin(10μM) ($P < 0.05$) (Fig. 4A, B). The same phenomenon was observed in KGNs (Fig. 4C, D).

SESN2 knockdown partly inhibited the antiapoptosis effect of melatonin in HUVECs incubated with angiotensin II

To further confirm the function of SESN2 during OHSS, we downregulated SESN2 levels by *siSESN2* (Fig. 5A, B). SESN2 knockdown significantly decreased the level of p-AMPK and increased the levels of p-mTOR and its downstream molecules (p-S6K1, p-4EBP1) ($P < 0.05$) (Fig. 5C, D), which revealed that SESN2 may be the upstream regulator of AMPK and mTOR. But the expression of BCL2 and p17 caspase 3 showed no difference after SESN2 knockdown. Treatment with melatonin and then knockdown SESN2 in HUVEC OHSS model also partly reduced its antiapoptosis effect by increasing p17 caspase 3 and decreasing BCL2 compared to that in non-SESN2 knockdown HUVEC OHSS oxidative models($P < 0.05$) (Fig. 5A, B). Thus, we speculated that the antiapoptosis effect of melatonin might be regulated through SESN2-AMPK-mTOR signaling pathway.

mTOR inhibitor rapamycin partly decreased the antiapoptosis effect of melatonin in HUVECs incubated in angiotensin II

After incubation with rapamycin, the BCL2 level was significantly decreased in HUVECs treated with melatonin, while there was no significant difference in SESN2 and p-AMPK levels. However, the p-mTOR and its downstream molecules (p-4EBP1 and p-S6K1) were significantly decreased after incubation with rapamycin, which revealed that SESN2 and AMPK may be the upstream regulator of mTOR, and mTOR plays an important role in the regulation of the antiapoptosis function of melatonin. (Fig. 6A, B).

Discussion

In this study, we demonstrated that SESN2 level was increased in the granulosa cells of OHSS patients and was involved in the oxidative stress of OHSS by regulating the apoptosis of endothelium cells. Incubation with angiotensin II in cultured HUVECs and H_2O_2 in cultured KGNs induced augmented ROS generation and increased SESN2 expression, both of which were restored by the treatment of melatonin. These beneficial effects of melatonin could be explained partly by regulating antiapoptosis through SESN2-AMPK-mTOR.

OHSS is associated with more morbidity and mortality than other complications during IVF process. Despite strides to reduce the incidence of this potentially fatal and completely iatrogenic complication, it remains a serious health concern for a significant percentage of patients undergoing IVF[McDonough et al.,2003]. The overabundance of ROS released by VEGF and inflammatory factors causes the dilatation of endothelium, which leads to a massive shift of the body fluids from the vessels into the third space and increases the severity of inflammation and tissues injuries during the process of OHSS. Higher levels of melatonin are found in human follicular fluid than in plasma because melatonin is not only derived from the general circulation but also synthesized in the ovary [Brzezinski et al.,1987]. Melatonin and its metabolites are free radical scavengers. Therefore, we speculate that the role of melatonin in the follicular fluid is to protect the oocytes and granulosa cells from being deteriorated by the oxidative stress and free radicals.

OHSS is triggered by ovarian hyperstimulation and characterized by arteriolar vasodilation and increased vascular permeability, resulting in systemic circulation changes that lead to salt and water retention. Ovaries are the source of this disease and endothelium is the target which releases large amounts of molecules during the pathophysiological process of OHSS. The stimulation and deterioration of vascular endothelial cells caused by ROS are the critical factors of OHSS. We found out that SESN2 level was closely associated with ROS, while melatonin could ameliorate SESN2 level induced by angiotensin II or H_2O_2.

SESN2 is a cytoplasm stress associated protein that accumulates in cells exposed to hypoxia, stress and DNA damage. Here, we demostrated significantly higher level of SESN2 was expressed in high risk OHSS patients compared to that in controls,which may be attributed to an increased oxidative stress in OHSS patients.These results are constant with previous studies which showed that any condition which leads to ROS accumulation may induce SESN2 expression[Lee et al.,1998]. Oxidative stress drives endothelium deteriotation, which contributes to the development and progession of OHSS. Anti-inflammatory and antioxidant agents prevent endothelium dilatation and reduce the oxidative imbalance in OHSS. Melatonin, as one of the most powerful free radical scavengers prevents granulosa cells in ovaries and endotheliums from the deteriotation of these free radicals.

The mechanisms by which melatonin provide antioxidant protection in OHSS are not fully understood. Previous studies have demonstrated that melatonin regulates antiapoptosis pathway[Ma et al.,2018;Shi

et al.,2018].BCL2 family and caspase family are important for regulating the atresia of antral follicles. Deletion of BCL2 increases the apoptosis of preantral follicle while the overexpression of caspases results in increased number of apoptotic follicles[Brzezinski et al.,1987]. Our study demonstrated that p17 caspase 3 level was increased while BCL2 level was decreased in OHSS oxidative models, which testified that the apoptosis signaling pathway was activated in OHSS oxidative models. Melatonin, could partly reverse p17 caspase 3 and BCL2 levels in OHSS oxidative model, which revealed the antioxidant effect of melatonin in OHSS by preventing oxidative stress-mediated apoptosis.

In recent years, more and more studies have shown that AMPK-mTOR molecules were also closely associated with apoptosis. Chen et al. have shown that H_2O_2 could induce apoptosis of neurons through activating AMPK and inhibiting mTOR[Wullschleger et al.,2006]. Arsikin et al. have also demonstrated that 6-hydroxydopamine induced apoptosis through AMPK in SH-SY5Y neuroblastoma cells [Arsikin et al.,2012]. Our study demonstrated that ROS could active SESN2-AMPK-mTOR which resulted in the apoptosis in OHSS oxidative models. Melatonin, by inhibiting apoptosis through regulating mTOR mediated pathway prevents the degenerescence disease in nervous system. Our study showed when SESN2 was knocked down, the antagonist effect against apoptosis by melatonin was inhibited, which revealed that SESN2-AMPK-mTOR played an important role in the antagonist process of apoptosis by melatonin. The same effect was shown by the incubation of mTOR inhibitor rapamycin, which testified again that mTOR played an important role in the regulation of apoptosis by melatonin. Meanwhile, there was no significant difference of p-AMPK levels by incubation with mTOR inhibitor in OHSS oxidative models, revealing that AMPK could be the upstream regulator of mTOR, which was in accordance with the previous reports[Bolotta et al.,2020]. There are also reports indicated that SESN2 knockdown decreased p-AMPK level and increased p-mTOR as well as its downstream molecules(p-4EBP1 and p-S6K1)[Ebnoether et al.,2017]. This may be explained that SESN2 was the upstream regulator.

Finally, the results described herein help us understand the beneficial effects of melatonin in OHSS patients. The antioxidative and antiapoptotic properties of melatonin seem to produce positive effects on OHSS. Considering the safety of exogenous melatonin has been testified in many studies [Jan et al.,2007], our present findings will provide us the potential for clinical application of melatonin to prevent OHSS and define the most appropriate timing when the administration of melatonin should be effectively carried.

Declarations

Ethics approval and consent to participate

Our research was approved by the Reproductive Ethics Committee of Ren Ji Hospital(No.2015030308). The informed consent was obtained from each patient before oocyte retrieval. We confirm that our methods were carried out in accordance wit the Helsinki Declaration.

Consent for publication

All authors agree this manuscript to be published.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that there are no conflict of interests.

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Author contributions

M.Z. and M.L. collected the samples. M.Z. performed the experiments, analyzed the data and wrote the paper. C.Z. designed the experiments, provided crucial suggestions. All authors have read and appoved the final submitted manuscript.

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Figures

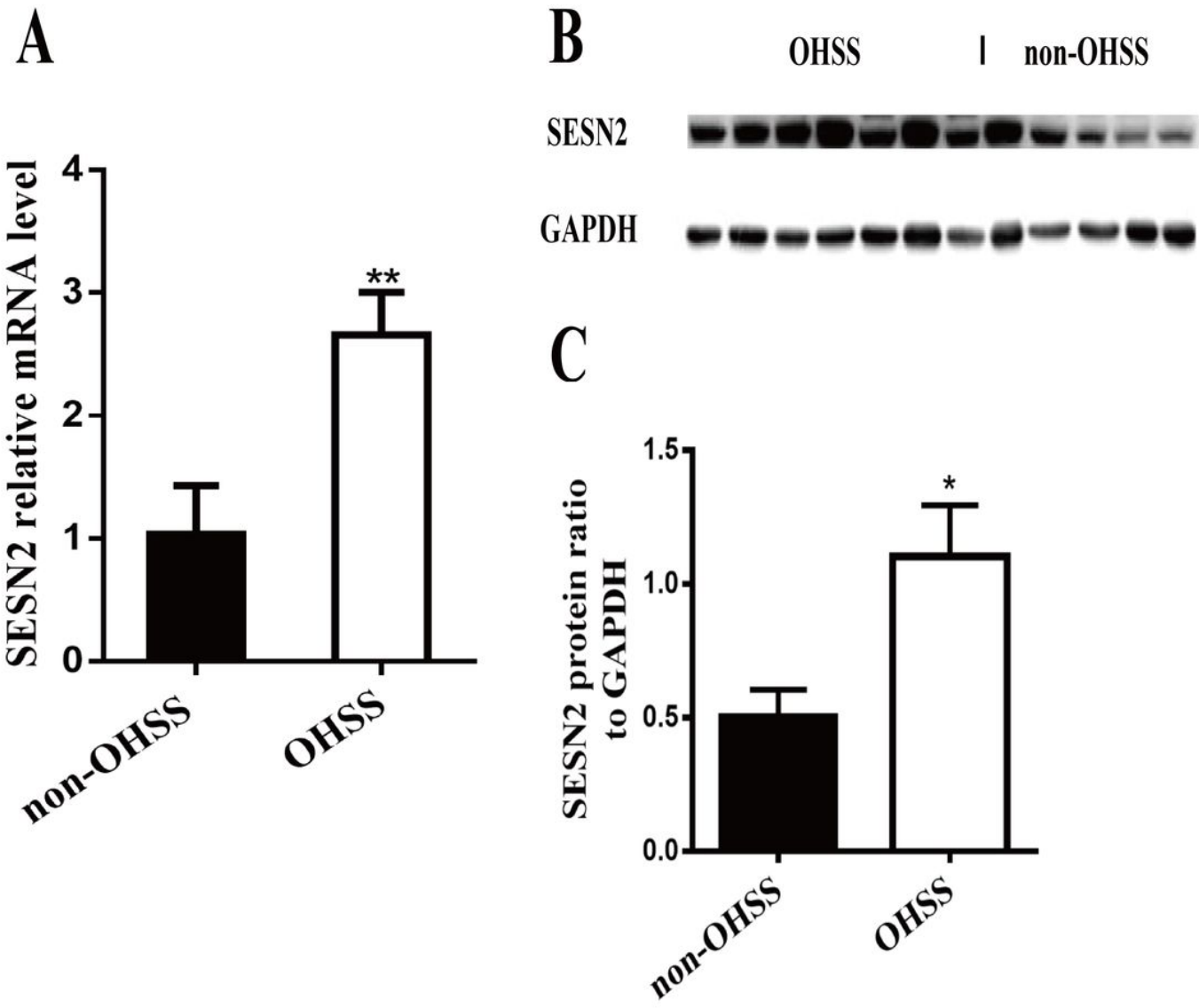

Figure 1

The expression of SESN2 in granulosa cells of OHSS patients and controls.(A) The expression of *SESN2* mRNA in granulosa cells of high risk OHSS(n=24) patients and controls (n=15) (t=-3.231, P=0.003); (B) The representative pictures of Western blotting of SESN2 and GAPDH in the granulosa cells of high risk OHSS patients and controls; (C) The comparison of SESN2/GAPDH protein ratio in the granulosa cells of high risk OHSS patients (n=24) and controls (n=15). *P<0.05;**, P<0.01.

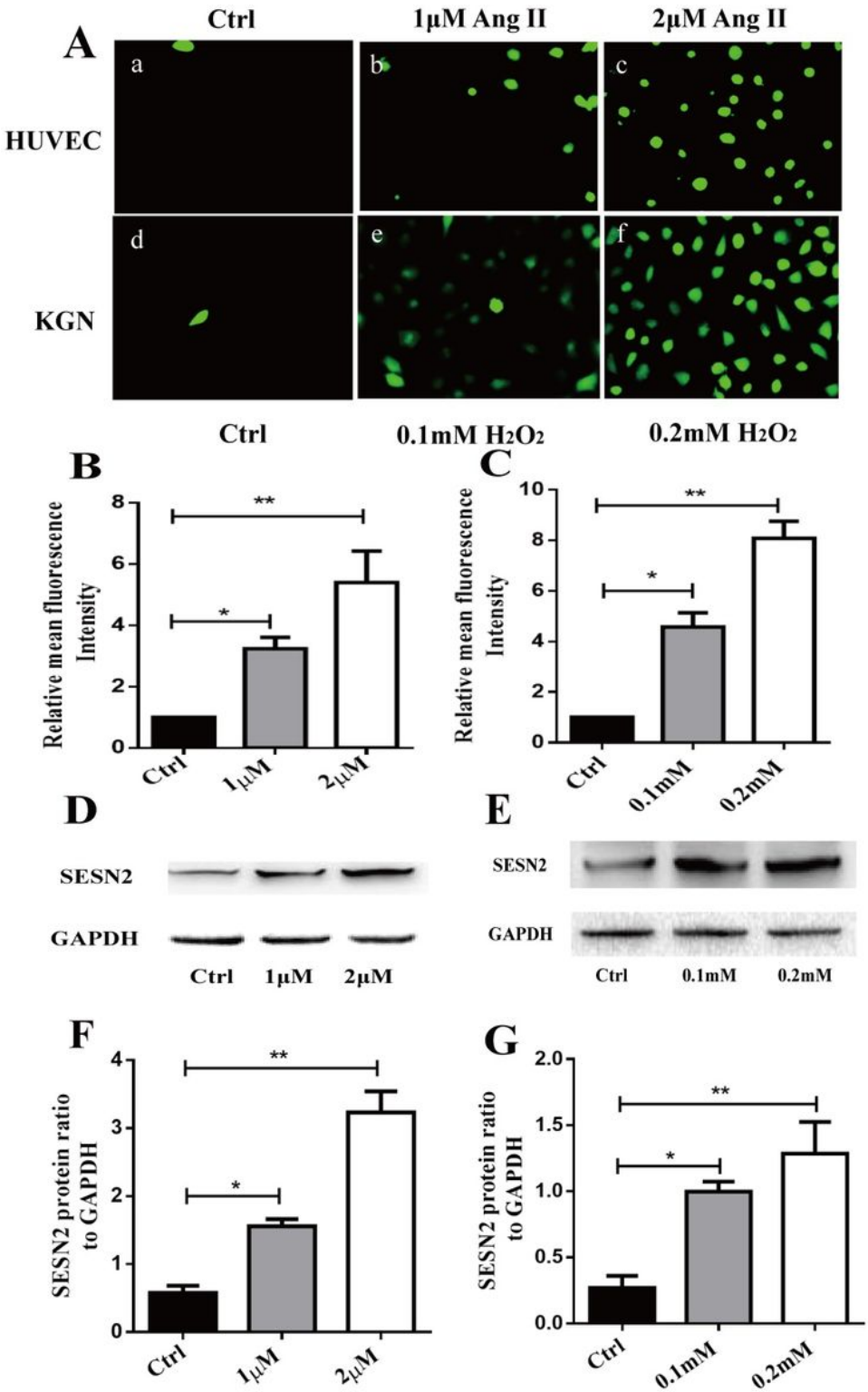

Figure 2

The oxidative stress and SESN2 levels in HUVECs and KGNs induced by different concentrations of angiotensin II or H_2O_2. (A) (a)(b)(c) The representative pictures of controls, 1μM, 2μM angiotensin II treated HUVECs dyed with DCFH-DA, respectively; (d)(e)(f) The representative pictures of controls, 0.1mM, 0.2mM H_2O_2 treated KGNs dyed with DCFH-DA, respectively; (B) The comparison of mean oxidative fluorescence intensity in HUVECs. The mean of the results in controls was assigned an arbitrary value of 1.0 and the results (mean ± SD) of other cells are expressed as the relative intensity; (C) The comparison of mean oxidative fluorescence intensity in KGNs. The mean of the results in controls was assigned an arbitrary value of 1.0 and the results (mean ± SD) of other cells are expressed as the relative intensity. Bar: 50μm; (D) The representative picture of Western blotting of SESN2 and GAPDH in controls, 1μM, 2μM angiotensin II treated HUVECs；(E) The representative picture of Western blotting of SESN2 and GAPDH in controls, 0.1mM, 0.2mM H_2O_2 treated KGNs; (F) The comparison of SESN2/GAPDH ratio after being incubated with angiotensin II for 24 h; (G) The comparison of SESN2/GAPDH ratio after incubation with H_2O_2 for 12 h. The results are expressed as mean ± SD. *，P<0.05; **, P<0.01; ***, P<0.001. Ctrl: control.

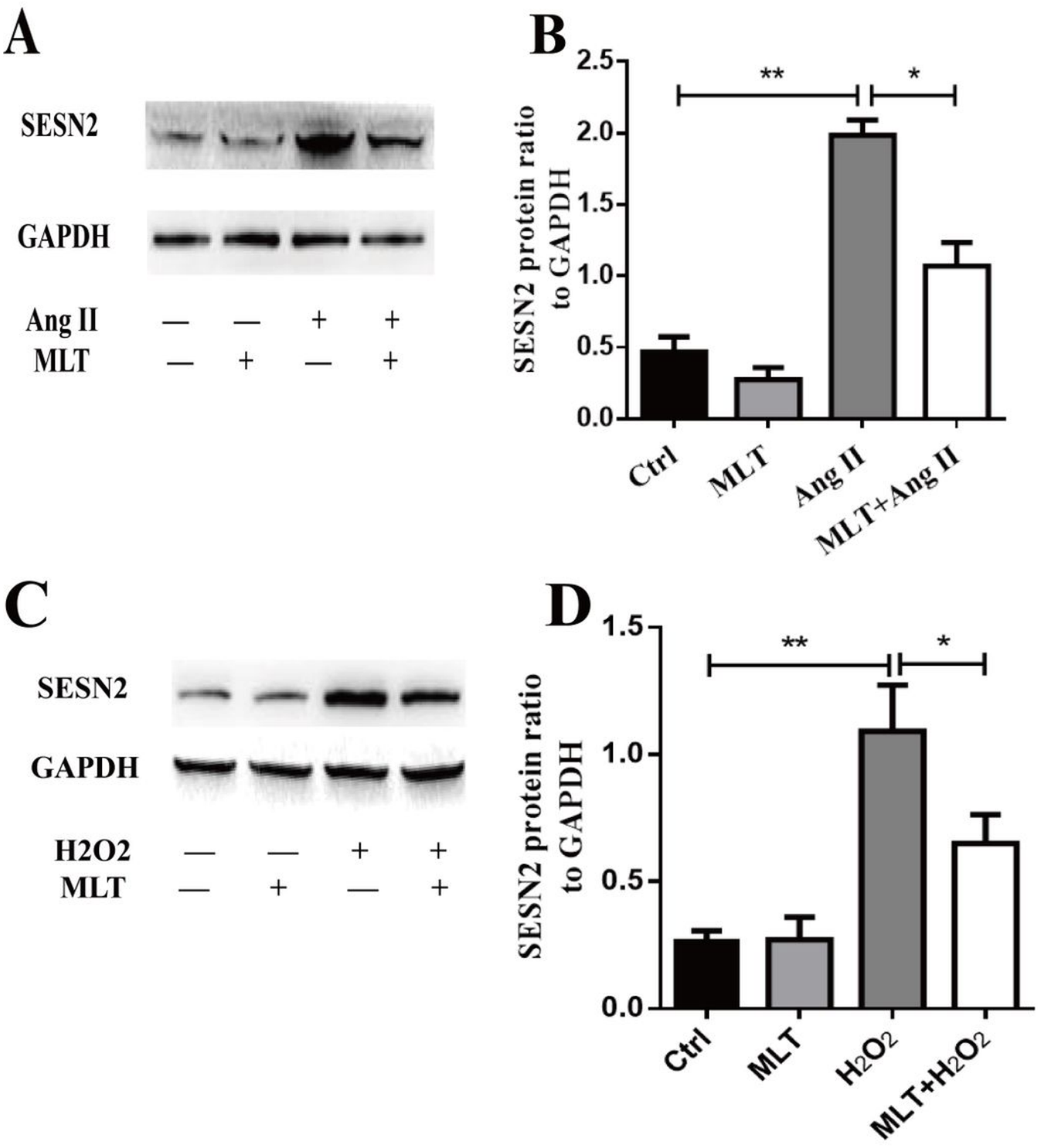

Figure 3

The effect of melatonin on SESN2 levels of HUVEC and KGNs incubated with angiotensin II or H_2O_2. (A) The representative pictures of Western blotting of SESN2 and GAPDH in controls, 10μM melatonin or 1μM angiotensin II treated cells before incubation with 1μM angiotensin II in HUVECs (B) The comparison of SESN2/GAPDH ratio in controls, angiotensin II, melatonin treated HUVECs; (C) The representative pictures of Western blotting of SESN2 and GAPDH in controls, 1mM melatonin, 0.1mM

H_2O_2, 1mM melatonin treated cells before incubation with 0.1mM H_2O_2 in KGNs (D) The comparison of SESN2/GAPDH ratio in controls, melatonin, H_2O_2, treated KGNs.The results are expressed as mean ± SD. *P<0.05; **, P<0.01; ***, P<0.001. Ctrl:control; MLT: melatonin; Ang II: angiotensin II.

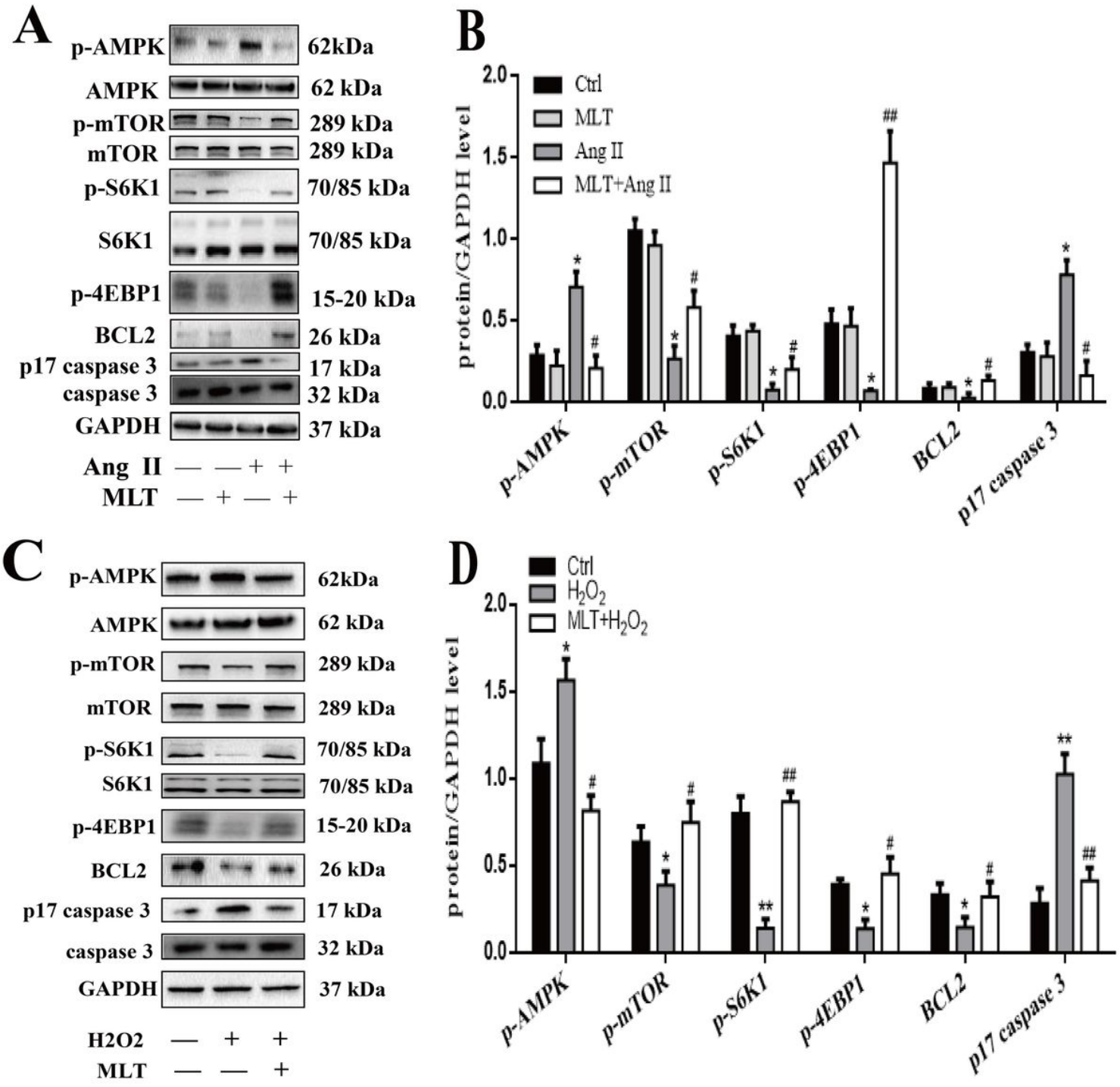

Figure 4

The effect of melatonin on apoptosis related proteins in HUVECs and KGNs incubated with angiotensin II or H_2O_2. (A) The representative pictures of Western blotting of p-AMPK, AMPK, p-mTOR, mTOR and its downstream factors (p-S6K1, S6K1, p-4EBP1) as well as apoptosis related molecules(p17 caspase 3,

caspase 3, BCL2) and GAPDH in melatonin and angiotensin II treated HUVECs; (B The comparison of protein/GAPDH ratio in in HUVECs; (C) The representative pictures of Western blotting of p-AMPK, AMPK, p-mTOR, mTOR and its downstream factors (p-S6K1, S6K1, p-4EBP1) as well as apoptosis related molecules(p17 caspase 3, caspase 3, Bcl2) and GAPDH in melatonin and H_2O_2 treated KGNs; (D) The comparison of protein/GAPDH ratio in KGNs. The results are expressed as mean ± SD. *P<0.05 vs. control group; **, P<0.01 vs. control group; #, P<0.05 vs. angiotensin II group; ##, P<0.01 vs. angiotensin II group. Ctrl: control; MLT: melatonin; Ang II: angiotensin II.

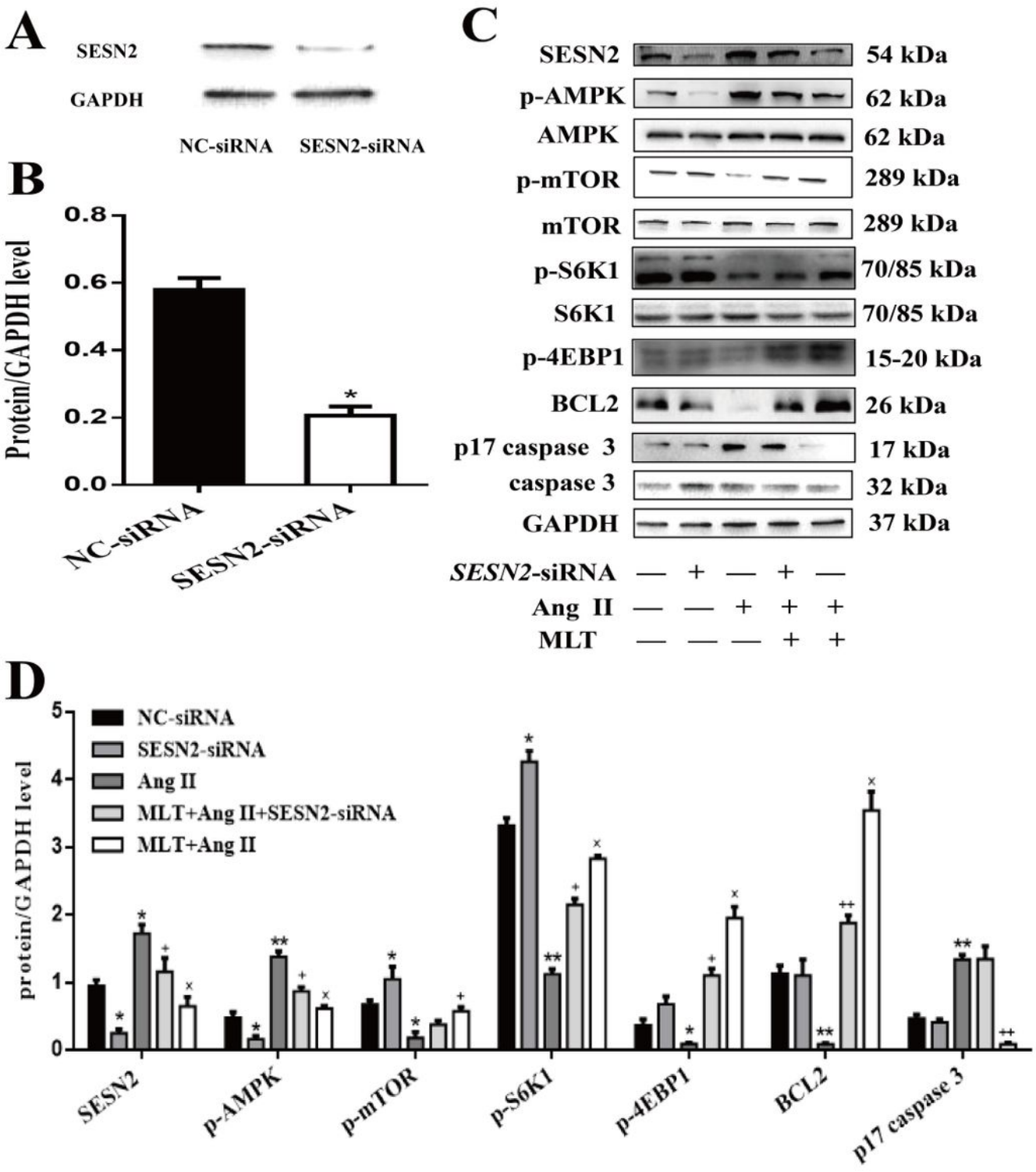

Figure 5

The effect of SESN2 knockdown in HUVECs incubated with melatonin and angiotensin II. (A) The representative pictures of Western blotting of SESN2 and GAPDH after transfection of siRNA(cropped gels)；(B) The comparison of SESN2/GAPDH ratio after transfection of siRNA in HUVECs (P=0.028); (C) The representative pictures of Western blotting of SESN2, p-AMPK, AMPK, p-mTOR, mTOR and its downstream factors (p-S6K1, S6K1, p-4EBP1) as well as apoptosis related molecules (p17 caspase 3, caspase 3, BCL2) and GAPDH in *SESN2-siRNA knockdown* and angiotensin II treated HUVECs(cropped gels)；(D) The comparison of protein/GAPDH ratio in controls, *SESN2-siRNA knockdown,* melatonin and angiotensin II treated cells. The results are expressed as mean ± SD. *, P<0.05 vs. control group; **, P<0.01 vs. control group; +, P<0.05 vs. angiotensin II group; ++, P<0.01 vs. angiotensin II group；×, P<0.05 vs. melatonin + angiotensin II + *SESN2*-siRNA group.

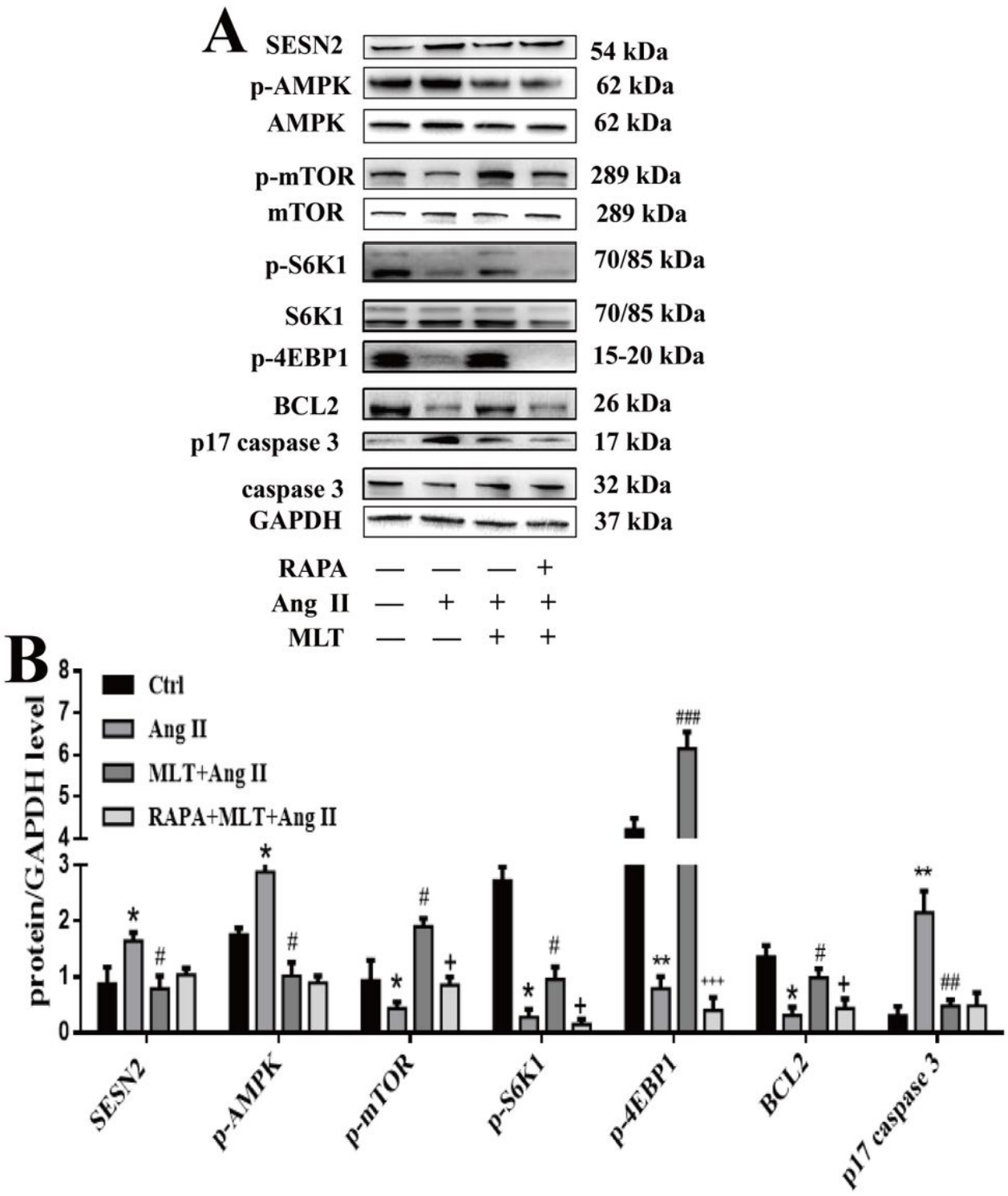

Figure 6

The effect of mTOR inhibitor rapamycin on HUVECs incubated with melatonin and angiotensin II. (A) The representative picture of Western blotting of SESN2, p-AMPK, AMPK, p-mTOR, mTOR and its downstream factors (p-S6K1, S6K1, p-4EBP1) as well as apoptosis related molecules (p17 caspase 3, caspase 3, BCL2) and GAPDH in angiotensin II, melatonin and rapamycin treated cells (B) The comparison of protein/GAPDH ratio in angiotensin II, melatoninand rapamycin treated cells. The results are expressed as

mean ± SD. *P<0.05 vs. control group; **, P<0.01 vs. control group; #, P<0.05 vs. angiotensin II group; ##, P<0.01 vs. angiotensin II group; +, P<0.05 vs.melatonin; ++ angiotensin II group; +++, P<0.001 vs. melatonin + angiotensin II group.