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Evaluation of the effects of angiotensin II and mitochondria transplantation on IL-1β, IL-6 and IL-10 cytokines in rat cardiomyoblast cells

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Angiotensin II (Ang II) causes mitochondrial dysfunction in the cardiovascular system, which is one of the underlying causes of cardiovascular pathologies. This study aimed to investigate the effects of Ang II and mitochondria transplantation on the proliferation, apoptosis, inflammatory and anti-inflammatory cytokines of rat cardiomyoblasts (H9c2). Mitochondria were isolated from rat mesenchymal stem cells (MSCs) with a commercial kit. Total protein and ATP levels were measured. Ang II (0.1 µM) and Mitochondria (5 µg/mL, 10 µg/mL) cytotoxicity was evaluated by MTT method. IL-1β, IL-6, IL-10 and caspase-3 levels were measured with ELISA. It was determined that decreasing doses of Ang II starting from 10 µM increased cell proliferation. H9c2 proliferation increased in the group that received Ang II (0.1 µM)+Mitochondria (10 µg/mL) compared to Ang II (0.1 µM) group. IL-6 levels showed a partial decrease with Ang II alone (0.1 µM) compared to the control. In addition, with the combined application of Ang II (0.1 µM) + Mitochondria (5 µg/mL), IL-6 levels decreased compared to both Ang II alone (0.1 µM) and the control group. On the other hand, it was found that IL-10 levels increased, on the contrary. These results suggest that mitochondria transplantation MT may enhance anti-inflammatory activity by Ang II type-2 receptors and affect in various apoptotic and proliferative pathways. Further studies are needed to elucidate the relationship between Ang II receptors and mitochondria on intracellular inflammatory signaling processes.

Keywords: Apoptosis, Inflammatory, Anti-inflammatory cytokines, Angiotensin II, Mitochondria isolation

Mitochondria have important roles in cellular functions including ATP production, calcium signaling, cell growth, cell proliferation and differentiation, reactive oxygen species production, cell cycle control, and cell death. Mitochondria are distinctive structures which contain maternally inherited DNA, are found in all mammalian cells except erythrocytes[1]. Mitochondria transplantation (MT) was first presented in 2009 by McCully *et al*. as a new tool for the treatment of ischemic diseases and obtained promising results, which showed an increased recovery rate for ischemia-reperfusion injuries[2,3].

In recent studies, MT has been investigated as a novel method for the treatment of multiple diseases associated with mitochondrial damage. Most studies have produced promising results for the treatment of ischemic heart diseases, stroke, and neurological degenerative diseases. Mitochondria can be isolated from various cell culture lines (e.g. mesenchymal stem cells, myocytes, fibroblasts), and non-autologous or autologous tissues (e.g. skeletal muscle, liver) by ultracentrifugation or with the aid of commercial kits[4-6].

MT can be performed by many different methods[4,5]. There are many animal studies which have tested their direct injection into the tissue and intravenous systemic administration. Furthermore, mitochondria can be added to the medium and thus be transferred by co-incubation in cell culture studies. It is reported that extracellular mitochondria pass into the cell within approximately 1-2 hours and mitochondria can enter the recipient cell through nanotubes, gap junctions, cell fusion, and extracellular vesicles[7]. They can also be transferred by microinjection, mitoception, co-incubation, peptide and polymer modification[8,9].

Ang II has very potent vasoconstrictor effects and is responsible for the control of body fluid volume, cardiac output, and regulation of blood pressure. It also induces proliferation, growth, increased fibrosis and inflammation in cells[10]. Ang II affects multiple organs and systems, especially the cardiovascular

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system. There are two types of Ang II receptors in tissues, namely, Ang II type-1 receptors (AT1) and Ang II type-2 receptors (AT2). AT1 receptors are predominant in adults and found in various tissues, including among others vascular, adipose and cerebral tissues. The best known biological effects of Ang II are mediated via AT1 receptors[11]. Recent studies on Ang II have shown that besides its vasoconstrictor effects, this signalling molecule may also bound up with inflammatory diseases. Ang II causes tissue damage by binding to AT1 receptors, triggering the generation of free oxygen radicals and causing mitochondrial dysfunction[10,12]. Contrary to these effects, the activation of AT2 receptors is known to induce vasodilation as well as anti-proliferative and anti-fibrotic outcomes by increasing nitric oxide (NO)[7].

Ang II causes mitochondrial dysfunction in the heart and vascular system and is also known to be one of the underlying causes of cardiovascular pathologies[13]. MT is performed to increase recovery in cardiovascular pathologies (ischemia, hypertension, atherosclerosis and myocardial infarction)[14].

In this study, we aimed to investigate the effects of MT, which were isolated from MSCs to Ang I I-administered cardiomyoblast cells (H9c2) on proliferation, apoptosis, and inflammatory and anti-inflammatory cytokines.

Materials and Methods

Cell culture

The rat cardiomyoblast cell line H9c2 (Rockville, MD, USA, ATCC# HTB 8 1) was obtained from Ankara University, and the rat MSCs line (SCR027-Millipore, Germany) was obtained from the the Gülhane Stem Cell Research Center of the University of Health Sciences, Türkiye. The cells were cultured in high-glucose (4.5 g/L) Dulbecco's modified Eagle's medium (Capricorn Scientific) supplemented with 10% fetal bovine serum (Capricorn Scientific), and 100 U/mL penicillin-100 µg/mL streptomycin (P4333-100 mL, Sigma-Aldrich) at 37°C in a 5% CO_2 atmosphere.

Determination of cell proliferation

H9c2 were seeded into 96-well culture plates (Costar, Cambridge, UK) at densities of 5000-10.000 cells/well and cultured for 24 hours. The cells exposed to 0, 0.001, 0.01, 0.1, 1, 10, 100, 1000 µM/L Ang II for 24 hours. After washing cells with fresh medium. Isolated mitochondria (5 µg/mL and 10 µg/mL) were added and cultured for 24 hours. After application of 100 µL MTT solution (0.5 mg/mL) to each well, the cells were incubated for another 2-4 hours. Subsequently, removing MTT in wells, 100 µL of DMSO (Merck, Germany) was added and 20-30 minutes was allowed for the dissolution of formazan. Finally, the absorbance value of each well was measured at 570 nm with a microplate reader (Molecular Devices Filter Max F5).

Mitochondrial isolation and transfer

MSCs were grown until 80-90% confluence in T75 flasks. Cells were counted with the trypan blue method using a hemocytometer. Mitochondria isolation buffer (1 mL) was added over 2×10^7 MSCs pellet, vortexed for 5 seconds and incubated for 2 minutes on ice. Mitochondria were isolated from rat MSCs using a mitochondria isolation kit (K288-50, Biovision Milpitas, CA USA) according to the manufacturer′s instructions. The isolated mitochondria were suspended in 0.5 mL of storage buffer. The holding time of mitochondria after isolation is a critical parameter. In our study, mitochondria were isolated from rat MSCs and kept on ice at +4°C during measurement of protein amount and ATP levels and transferred to H9c2 cells within approximately 1 hour.

Protein and ATP measurement

The protein concentrations of the isolated mitochondria and experimental groups of H9c2 were detected with the PierceTM BCA Assay Kit (23227, Thermo Fisher Scientific Inc., MA, USA). ATP measurement assay was based on luciferase′s requirement for ATP in producing light. ATP levels were determined by measuring the metabolic activity of the mitochondria isolated from MSCs using an ATP determination kit (A22066, Invitrogen, Molecular Probes) protocol.

IL-10, IL-6, and IL-1β determination by ELISA

Cell lysate samples were collected and centrifuged at 200×g. IL-10 (BMS629, eBioscience, Austria), IL-6 (BMS625, eBiocience, Austria) and IL-1β (KRC0011, Invitrogen, USA) levels were measured with ELISA kits following the manufacturer′s protocol. The absorbance of the samples was read with an ELISA reader at 450 nm. The data were normalised for protein content and expressed in pg/mg total protein.

Caspase-3 analysis

We used the Caspase-3/CPP32 Fluorometric Assay Kit (Biovision, K105-100) to evaluate apoptosis. Firstly, H9c2 cells were resuspended with cell lysis buffer (1× cell / 3× lysis buffer). It was used 50 µg cell lysates and added 2× reaction buffer (50 µL) to each sample. Next, 1 mM DEVD-AFC (5 µL) was added and incubated for 1-2 hours at 37°C. Each well was evaluated with a fluorometric plate reader using a 400 nm excitation filter and a 505 nm emission filter.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 program. Study data are expressed as mean±SE. Normality of distribution was tested using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) test was used for data with homogeneous variances, and Tukey post-hoc HSD test was used to evaluate statistical differences between groups. For data with non-homogeneous variances that did not comply with normal distribution, the Mann-Whitney U test was used after the Kruskal-Wallis test. $P<0.05$ was considered statistically significant and all experimental analyzes were performed in triplicate.

Results

Effect of Ang II on H9c2 cell proliferation

Different concentrations of Ang II (0-100 µM) were applied to the H9c2 cells for 24 hours. Accordingly, an increase of 32.76% and 34.23% in cell proliferation was observed at 10 and 1 µM Ang II concentrations, respectively. At 0.1 µM Ang II concentration, the increase in proliferation was found to be 36.41%. At 0.01, 0.001 and 0.0001 µM Ang II concentrations applied to H9c2 cells, partial changes occurred in proliferation and the increase amounts were found to be 35.46%, 36.46% and 38.40%, respectively. It was determined that decreasing doses of Ang II starting from 10 µM increased cell proliferation significantly ($P < 0.05$) (Fig. 1). On the other hand, the partial increase in cell proliferation after application of 100 µM Ang II was not statistically significant compared to the control group ($P> 0.05$). Ang II (0.1 µM) was selected for combined administration with mitochondria, in our study.

Effect of Ang II administration and MT on H9c2 cell proliferation

Ang II was administered at a concentration of 0.1 µM and mitochondria (5 µg/mL and 10 µg/mL) were transplanted to H9c2 cells for 24 hours. The selected dose of Ang II (0.1 µM) increased cell proliferation by 34% ($P < 0.05$). MT (5 µg/mL and 10 µg/mL) did not cause any significant change in cell proliferation. It was found that the combined application of Ang II (0.1 µM) and mitochondria (5 µg/mL) produced a partial increase in cell proliferation compared to the application of Ang II (0.1 µM) alone, but this change was not statistically significant ($P> 0.05$). On the other hand, the combined application of Ang II (0.1 µM) and mitochondria (10 µg/mL) produced a dramatic increase in cell proliferation compared to the application of 0.1 µM Ang II alone, and this change was found to be statistically significant ($P < 0.05$), (Fig. 2).

Mitochondrial protein and ATP levels

The ATP level of the isolated mitochondria was

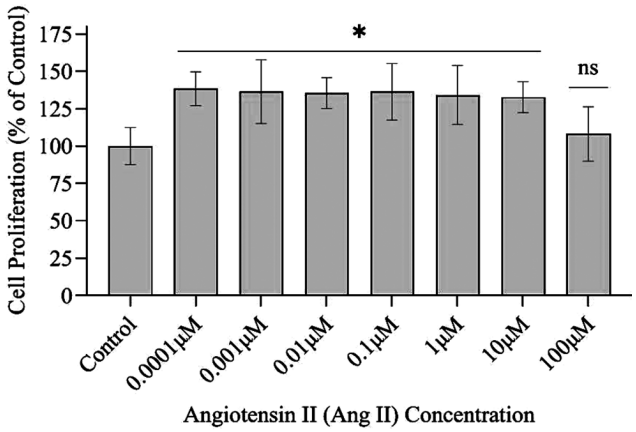

Fig. 1 — Change in H9c2 cells proliferation after Ang II application (after 24 h incubation), n=3.

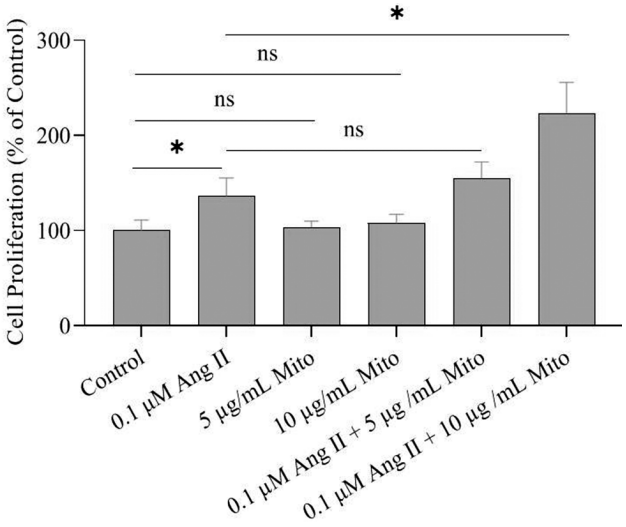

Fig. 2 — Synergistic effects of combined application of Ang II and mitochondria on H9c2 cells proliferation, n=3.

calculated from the standard curve, 8.298 nmol/mL, and the total protein level was 0.477 mg/mL. The ATP level was calculated as 17.36 nm ATP/mg protein.

Effects of Ang II and MT applications on caspase-3, IL-6, IL-1β and IL-10 levels in H9c2 cells

Ang II (0.1 μM) and MT (5 μg/mL) were applied to H9c2 cells, and after 24 hours incubation, caspase-3, IL-6, IL-1β and IL-10 levels were measured in cell extracts by ELISA. Based on the comparison of caspase-3 levels, no significant difference was determined between the groups (Fig. 3A.). There was a decrease in IL-6 levels in the Ang II (0.1 μM) group compared to the control group (P> 0.05). The group administered together with Ang II (0.1 μM) and mitochondria (5 μg/mL) had lower IL-6 levels compared to the Ang II (0.1 μM) group (P> 0.05), (Fig. 3B). IL-1β levels were attenuated in the Ang II (0.1 μM) and mitochondria (5 μg/mL) group compared to control group (P> 0.05), (Fig. 3C.). IL-10 levels increased in the Ang II (0.1 μM) group compared to the control group. In addition, the group administered together with Ang II (0.1 μM) and mitochondria (5 μg/mL) had higher IL-10 levels compared to the Ang II (0.1 μM) group, but these changes were not statistically significant. (P> 0.05) (Fig. 3D).

Discussion

In the present study, we demonstrated the effects of Ang II and MT on cardiomyoblasts. In the dose application of Ang II alone in H9c2 cells, it was seen that doses lower than 100 μM increased cell proliferation. A concentration of 0.1 μM of Ang II has been used as a proliferative and hypertrophic dose for combined treatments in several previously conducted model studies[15]. In line with the findings we obtained after our cell proliferation study and the literature, we choose the Ang II dose as 0.1 μM for combined applications with MT. Our study demonstrated that the combined administration of Ang II and MT significantly increased H9c2 proliferation. Many animal and human studies have shown that MT prevents cell damage, enables cell repair and increases ATP production in cardiovascular disease models associated with mitochondrial dysfunction[16]. In this study, the results obtained by applying Ang II in combination with different doses of mitochondria showed that MT at 10 μg/mL caused a greater increase in H9c2 proliferation than MT at 5 μg/mL.

Different values have been reported for different levels of MT in previous studies[16,17]. In pediatric infants with congenital ischemic heart disease; mitochondria isolated from autologous skeletal muscle were directly injected into the pericardium at a dose of 2×10^5 mitochondria per gram of tissue, such that an improvement was detected in cardiac functions after MT[18]. In another study, mitochondria isolated

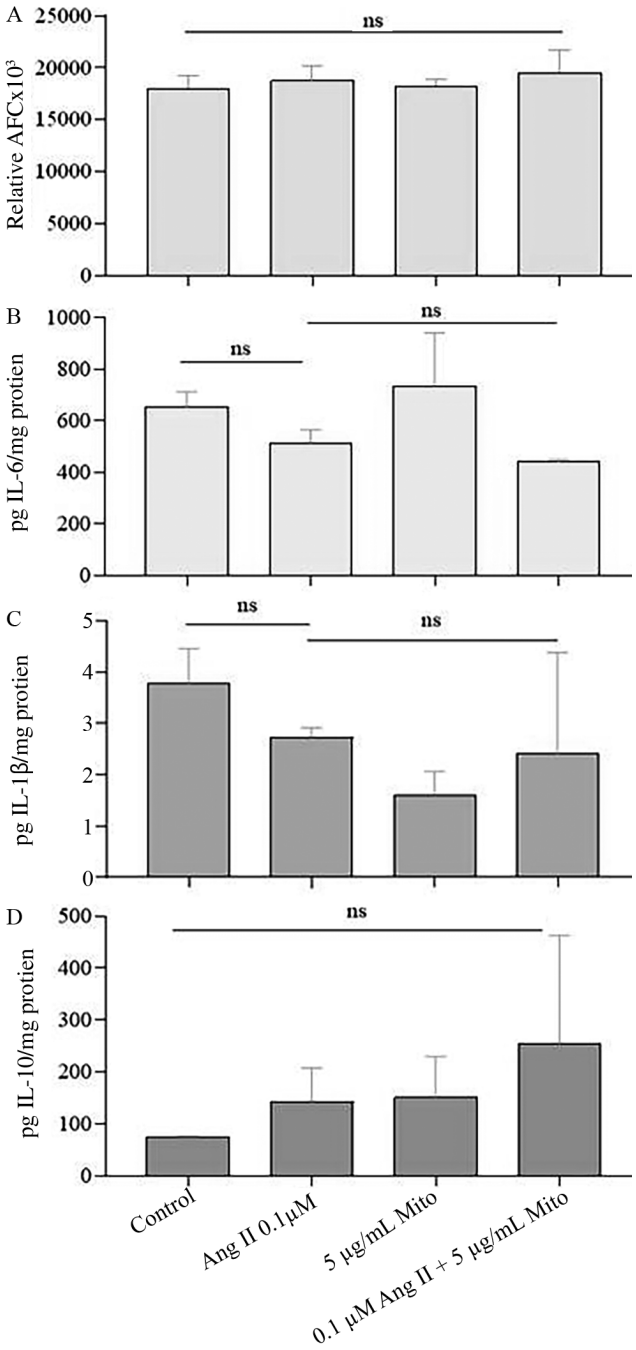

Fig. 3 — Caspase-3, IL-6, IL-1β and IL-10 levels in H9c2 cell line after Ang II and MT applications, n=3.

from autologous skeletal muscle were transferred by injection, in a regional ischemia model of the pig heart. However, no significant difference was observed in terms of the healing of the ischemic area between mitochondrial transplantations performed at doses of 2×10^5, 2×10^6, 2×10^7, and 2×10^8 mitochondria per gram tissue. Nonetheless, it was shown that autologous mitochondrial transplantation could be an effective cardioprotective treatment method[17]. In another in vitro study, mitochondria were isolated from human mesenchymal stem cells, the amount of protein was measured by the Bradford method, and 5 µg/mL of mitochondria were transferred to 20.000 MDA-2B-231 cells/well[19]. We transplanted 5 and 10 µg/mL of mitochondria to 10.000 H9c2 cells/well.In this study, we transplanted 5 and 10 µg/mL of mitochondriaisolated from rat MSCs were transplanted to 10.000 H9c2 cells/well.

One of the limitations of our study is the determination of the amount of mitochondria to be transplanted. It has been observed in the studies that mitochondrial transfer is done both by counting and by the amount of protein[16]. In this study, we preferred to measure the protein amount of the isolated mitochondria and it was confirmed that our mitochondrial treatment doses provided sufficient efficacy.

Another important limitation of this study is that the isolation process is critical for obtaining live and healthy mitochondria for the success of transplantation. The transplantation period should not be extended to maintain the stability of the inner and outer mitochondrial membranes[20]. Therefore, it is vital that mitochondria are transferred immediately after they are isolated from tissues or cells. Mitochondria transfer should be done with a waiting period not exceeding 2 hours. A longer waiting period reduces the loss of mitochondrial activity and viability[16,20,21]. In this study, mitochondria isolated from rat MSCs were stored at +4 °C during the measurements and applications and the waiting period was approximately 1 hour. The ATP level calculated for the isolated mitochondria (17.36 nm ATP/mg protein) confirmed that they were functional and sufficient, and also correlated with literature data[19,22].

In the present study, the evaluation of the effects on apoptosis, based on the caspase-3 pathway, demonstrated that neither combined nor individual Ang II administration and MT showed efficacy. Considering the cell proliferation-enhancing effects of MT, its correlation with the apoptosis mechanism could not be determined in this study. Zhou *et al*. investigated the antioxidant effects of epigallocatechin-3-gallate (EGCG) on HUVEC cells treated with Ang II. These researchers showed that Ang II increased the generation of free oxygen radicals, and thereby, led to cytochrome c release and caspase 3/9 activation[23]. Differently, in our study, no change was observed in caspase-3 activity. The differences between the two studies could be due to differences in the cell lines and Ang II doses used. In another Ang II-induced apoptosis study on the HUVEC cell line, Du *et al*. determined an increase in caspase-3 levels, similar to the study of Zhou *et al*.[23,24]. It was concluded that cell lines play a more active role and selected cell lines should be examined separately at the receptor level. In a study by Fang *et al*., MT was performed for the treatment of neurological damage caused by spinal cord ischemia[25]. These researchers determined a decrease in IL-6, TNF-α and caspase-3 levels. In our study, no decrease was determined to have occurred in caspase-3 levels with MT. The difference between the two studies was attributed to co-administration with Ang II.

The decrease determined in the present study in IL-6 levels, as a result of combined Ang II administration and MT, is similar to the results of Fang *et al*. In another study by Li *et al*., Ang II-induced inflammation and ferroptosis were investigated in astrocytes, and it was shown that IL-6 and IL-1β levels had increased with Ang II administration[26]. In our study, based on IL-6 measurements, it was ascertained that Ang II administration alone reduced inflammation. MT alone did not alter the IL-6 level, and Ang II administration reduced inflammation with a synergistic effect. Our results suggest that, while MT inhibits inflammation, it activates anti-inflammatory processes. In this study, no statistically significant difference was determined between the study groups for IL-1β levels, and IL-1β levels decrease in the groups treated with Ang II and Ang II+MT groups. The anti-inflammatory effect of Ang II and its synergistic effect with MT are considered to be dose-dependent. The difference between the present study and the study of Li *et al*. is attributed mainly to the Ang II dose used and the different Ang II receptor (AT1 and AT2) responses elicited by the use of a different cell line such as astrocytes. Other studies using higher Ang II doses

have reported inflammatory effects. In addition, our results could be attributed to the co-administration of Ang II at a dose of 0.1 μM and mitochondria at a level of 5 μg/mL reducing inflammation through the inhibition of free oxygen radical generation. In their study on the correlation between Ang II and IL-6 in astrocytes, Gowrisankar & Clark found that Ang II induced IL-6 production in astrocyte cells[27]. This result supports the idea that the inflammatory effects of Ang II receptors could vary with cell types. Upon examining the interaction of IL-1β and AT1, Liu *et al*. showed that this interaction in the paraventricular nucleus of the hypothalamus increased the risk of heart failure. While Liu *et al*. investigated the effects of IL-1β on the hypothalamus, they did not examine cardiac receptors, but rather examined markers at a more systemic level. To our knowledge, there is no previous study on IL-1β levels after MT. In our study, IL-10 levels were measured, and it was determined that anti-inflammatory activity had increased in the Ang II and Ang II+MT groups. Our study showed that the Ang II+MT groups differed from the Ang II group, and that MT increased anti-inflammatory activity. In a study by Kwon *et al*., Ang II was injected to mice subcutaneously, and it was observed that IL-10 gene expressions increased in heart cells[28]. In our study, H9c2 myocyte cells were used, and Ang II similarly caused an increase in IL-10 levels. In a study by Bressan *et al*., exogenous IL-10 was shown to modulate ERK1/2 activation and prevent hypercontractility in an Ang II-induced hypertension model[29]. Although IL-10 was administered exogenously, it seems logical that cells increase the production of endogenous IL-10 against Ang II, given its efficacy on Ang II[29]. These results are in agreement with ours. Lima *et al*. injected Ang II to mice, and investigated effects on blood pressure and the RhoA7Rho-kinase signaling pathway. These researchers determined that IL-10 inactivated the repressive activity of Ang II by partially modulating the RhoA/Rho kinase signaling pathway[30]. The results of these studies support our findings; such that IL-10 is thought to act not only as an anti-inflammatory agent, but also as a multi-functional cytokine. Not only does IL-10 show this activity in cardiac cell types, but it also blocks the effects of Ang II in neurons. In a study by Jiang *et al*., it was shown that the excitatory effects of Ang II on the ATR1 receptors in the paraventricular nuclei of the hypothalamus were prevented by the inhibitory effects of IL-10 in the Ang II-induced rat hypertension model. Thus, it is suggested that IL-10 shows a multisystemic activity against Ang II. There are not many studies on MT with IL-10. The critical control role of IL-10 on the immune system and its effects on mitochondria give us an idea on this subject[31]. Ip *et al*. showed that the anti-inflammatory effects of IL-10 are regulated by the metabolic reprogramming of macrophages. These researchers stated that IL-10 regulates mitophagy by preventing the accumulation of dysfunctional mitochondria[32]. Furthermore, the inflammatory and anti-inflammatory cytokine levels determined in our study demonstrated that Ang II administration and 0.5 μg/mL MT reduced inflammation and increased anti-inflammatory activity.

In the present study, while the cell type from which mitochondria were isolated belonged to the same species these organelles were transferred to, mitochondria isolated from the cells of a different species were used in previous research[16]. Literature review has shown that MSCs are one of the most preferred cell groups for mitochondrial isolation. The renewal ability and mitochondrial functions of MSCs provide more meaningful and successful results for transplantation compared to other cells[33].

Moreover, we showed the dose-dependent effects of Ang II, which is an inflammatory molecule capable of affecting many systems, especially on the cardiovascular system. We determined that while a low dose of Ang II reduced inflammation and did not affect the apoptosis pathway via caspase-3, MT caused a slight increase in apoptosis, but did not induce any significant change. It is considered that MT affects the signaling of different apoptotic and cell death pathways.

Conclusions

In conclusion, it has been reported the mechanisms underlying the therapeutic effects of MT are not fully understood yet and still not clearly well known through which receptors that affects inflammation or anti-inflammatory mechanisms. Additionally, MT can be considered to attenuate inflammation and increase anti-inflammatory activity by stimulating AT2 receptors. This is thought to be because ATR1 receptors activate inflammation compare to ATR2 receptors. However, much less is known about the mechanisms of action of ATR2 receptors in inflammatory processes. In addition, the viability and preservation of function of

isolated mitochondria depend on many factors such as isolation protocol, temperature and duration, which makes interlaboratory standardisation difficult. There is no accepted dosing criterion for MT; different applications are made according to the amount of protein or the number of mitochondria, which limits the comparability of the results.

Further studies are needed to elucidate the relationship of Ang II receptors and mitochondria on the intracellular inflammatory signaling processes and there is a need for various *in vitro* and clinical studies in which these molecular pathways are investigated in more detail.

Conflict of Interest:

No conflict of interest is declared by the authors.

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