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Momordica charantia reduced ovarian ischemia - reperfusion injury by suppressing APAF-1 expression

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Abstract. – OBJECTIVE: We investigate the effect of *Momordica charantia* (MC) against ovarian ischemia-reperfusion injury (IRI).

MATERIALS AND METHODS: Forty-eight Sprague Dawley female rats were divided into 6 groups. 3 hours ischemia/3 hours reperfusion was conducted. Before and/or after IR, 600 mg/kg MC were introduced to rats *via* an orogastric tube. At the end of the experiment, total serum antioxidant/oxidant status (TAS/TOS) and Anti-Mullerian Hormone (AMH) level were measured. Ovarian histopathology and APAF-1 expression level were analyzed.

RESULTS: TAS and AMH levels were the lowest, while TOS level and OSI were the highest in the IR group. TAS and AMH levels were higher, and TOS levels and OSI were lower in MC-treated groups compared to IR group. Follicular degeneration, granulosa and stromal cell degeneration, mononuclear cell infiltration and vascular congestion and dilatation were observed in the IR group. Ovarian histopathology was improved in groups that received MC extract. APAF-1 immune activity was intense in IR and MC+IR groups while decreased in the groups treated with MC extract after IRI. MC treatment downregulated APAF-1 protein after IRI.

CONCLUSIONS: MC extract restored negative biochemical and histochemical changes caused by IRI due to its antioxidant properties and supported cell survival by suppressing APAF-1 expression.

Key Words:
Ovary, Ischemia, Reperfusion, *Momordica charantia*, APAF-1.

Introduction

Ischemia-reperfusion injury (IRI) is a clinical emergency developed by myocardial infarction, acute heart failure, cerebral dysfunction, organ transplantation, stroke, peripheral vascular diseases, hypovolemic shock and multiple organ dysfunction[1]. IRI may develop in many organs such as heart, lung, kidney, intestine, skeletal muscle, and brain, and can also cause distant organ damage. As a result of IRI, local inflammation, production of reactive oxygen species (ROS), and many other cellular processes eventually lead to cell damage and cell death by apoptosis or necrosis[2]. There have been many studies on apoptosis and IRI in recent years. Oxidative stress and ROS production increases in IRI, leading to apoptosis[3].

Momordica charantia (MC, bitter gourd) is a plant belonging to the Cucurbitaceae family and grown in tropical and subtropical regions (especially in Asia, Amazon, East Africa and the Caribbean). It is used as a traditional medicine in the treatment of many medical diseases such as bacterial and viral infections[4,5]. Besides its antidiabetic properties, studies[6] showed that MC has anti-inflammatory, antioxidant, antiviral, antibacterial, anthelmintic, and anticancer activities due to its powerful bioactive compounds. MC powder reduced TNF-α and IL-6 levels in the blood and improved systemic inflammation and colon functions by regulating the expression of inflammatory markers in obese mice fed on a high-fat diet[7]. Pretreatment of MC extract in mice treated with TNF-α decreased cell death and inflammation, by suppressing the inflammatory pathway[8]. Kim et al[9] investigated the possible antioxidant activity of MC extracts in neuroblastoma cell culture *in vitro*. They showed that pre-administration of MC extract eliminated reactive oxygen species and reduced cytotoxic oxidative stress *via* p38 and ERK1/2 MAPK signaling pathway. Triterpene glycosides and triterpenoids isolated from the MC plant

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inhibited the activity of xanthine oxidase, removed ROS by inhibiting lipid peroxidase due to its antioxidant properties[10].

The aim of this study is to investigate the effects of MC extract with its antioxidant properties in the experimental ischemia-reperfusion model in the rat ovary.

Materials and Methods

Animal experiments were conducted by permission of the Dicle University Animal Experiments Local Ethics Committee (record number: 2020/08). Financial support for this study was provided by Dicle University Scientific Research Projects Unit (protocol number: TIP.20.009).

Ethanolic Extract Of MC

Powder form of sun-dried *Momordica charantia* fruit was obtained from a local seller. The powder was mixed with absolute alcohol (100% ethanol, catalog no: K49284483729, Merck, Germany) and stirred for 3 hours. The mixture was allowed to rest for 8 hours. The supernatant was taken and the procedure was repeated 3 times. Pure alcohol was evaporated and ethanolic extract of MC was obtained.

Experimental Procedure

48 female Sprague Dawley rats (8 rats per group, 10-12 weeks old, 250-300 g) were fed with normal diet and water at 24±1ºC in a 12-hour light/12-hour dark light period. All procedures were conducted under general anesthesia. Atraumatic stainless steel sterile Bulldog forceps were used for the IR.

- Sham group: No treatment was applied to the animals. Only the abdomen was opened with a surgical protocol and closed without any further intervention.
- MC group: Unlike the sham group, the animals were given 600 mg/kg MC extract by orogastric route for 15 days.
- IR group: Bilateral 3-hour ischemia 3-hour reperfusion was applied to ovaries.
- MC+IR group: The animals were administered with 600 mg/kg MC extract by orogastric route for 15 days. At the end of the 15th day, the IR procedure was performed.
- IR+MC group: The animals were given 600 mg/kg of bitter melon extract by orogastric route for 15 days after IR.
- MC+IR+MC: 600 mg/kg of bitter melon extract was administered orogastrically for 15 days before and after the IR procedure. At the end of experiment, all animals were sacrificed under anesthesia.

Serum TAS, TOS and AMH

Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) parameters were measured from serum according to the modified Erel method. TAS and TOS kits were purchased commercially (Rell Assay Diagnostics, Gaziantep, Turkey). Oxidative Stress Index (OSI) was calculated by division of the TOS/TAS[11]. Anti-Müllerian Hormone (AMH) levels were measured by commercially available rat compatible enzyme-linked immunosorbent assay kit (ELISA) (catalog number: MBS2509909, MyBioSource, CA, USA).

Histological Tissue Processing

Right ovarian tissues were processed for routine paraffin wax tissue embedding. Ovaries were fixed in zinc formalin fixative (catalog no: Z2902-3.75L, Sigma, Germany) solution, dehydrated in ascending alcohol series and embedded in paraffin blocks. Hematoxylin-Eosin and APAF-1 immunohistochemistry staining was applied to 5 µm thick sections[12,13].

Immunohistochemical Staining

Sections were brought to distilled water and treated with 1% hydrogen peroxide solution (cat#: TA-015-HD, ThermoFischer, CA, USA) to block the endogenous peroxidase activity. Sections were washed with Phosphate-buffered with Tween20 (PBST) 3×5 minutes, then blocked with Ultra V Block (cat#: TA-015-HD, ThermoFischer, CA, USA) to prevent non-specific binding. Sections were incubated overnight at +4°C with anti-APAF-1 (catalog no: ab2000, Abcam plc, Cambridge, UK) primary antibody. Biotin-containing secondary antibody (cat#: TA-015-HD, ThermoFischer, CA, USA) was dripped onto the sections and washed with PBST. Streptavidin-peroxidase (cat#: TA-015-HD, ThermoFischer, US) was dropped on slides and then washed with PBST. Diaminobenzidine (DAB) (cat#: TA-015-HD, ThermoFischer, CA, USA) was used as a chromogen to observe expression. Slides were counterstained with Gill III hematoxylin (catalog no: HX84908574, Mer-

ck, Darmstadt, Germany). Sections were passed through increasing alcohol series and cleared in xylene and analyzed with Motic EASYSCAN PRO 6 Pathology Slide Scanner (Meyer Instruments Inc, Houston, Tx, USA)[14-16].

Statistical Analysis

Statistical analysis of TAS, TOS, OSI and AMH data of all experimental groups were analyzed using IBM SPSS Statistics 25 (IBM Corp., Armonk, NY, USA) software. ANOVA and post-hoc Tukey and Games-Howell tests were used depending on the homogeneity of the variance. $p<0.05$ was considered statistically significant.

Results

TAS, TOS and AMH Findings

The mean±standard deviation, and statistical analysis of TAS, TOS, OSI and AMH values in the groups are shown in Table I. A significant decrease was observed in the mean TAS values in the IR group compared to the sham group ($p=0.003$). There was a non-significant increase in the mean TAS values in the MC+IR, IR+MC, and MC+IR+MC groups compared to the IR group ($p=0.959$, $p=0.113$, $p=0.296$, respectively). The mean TOS values were measured significantly higher in the IR group compared to the sham group ($p=0.001$). A significant decrease in the mean TOS values was observed in the IR+MC and MC+IR+MC groups compared to the IR group ($p=0.001$ and $p=0.001$, respectively). The mean OSI values in the IR group were significantly greater than sham group. Administration of MC extract reduced oxidative stress, but it was effective especially after IRI. Mean AMH values in the IR group were lower than the sham group. AMH levels increased in the MC+IR and IR+MC groups compared to the IR group, but it was significant in the MC+IR+MC group ($p=0.001$). MC treatment restored ovarian reserve by increasing AMH values in IRI, but pre- and post-IR administration was more effective.

Histopathological Findings

Hematoxylin-Eosin staining, and APAF-1 immunostaining of ovarian sections were shown in Figure 1. Ovarian histology looked normal in the sham group (Figure 1a) and MC group (Figure 1b). Follicular degeneration, intense vascular congestion and dilatation, and mononuclear leukocytes were observed in the IR (Figure 1c) and MC+IR (Figure 1d) groups. Pathologies were improved in IR+MC (Figure 1e) and MC+IR+MC group (Figure 1f).

Immunohistochemical Findings

Ovarian sections with APAF-1 immunohistochemistry staining were shown in Figure 2. Weak APAF-1 expression was observed in the sham and MC group (Figure 2a and 2b, respectively). APAF-1 immune activity was intense in the IR group (Figure 2c) and MC+IR group (Figure 2d). The APAF-1 reaction was mostly negative in ovarian follicles of the IR+MC group (Figure 2e) and MC+IR+MC group (Figure 2f). MC treatment strongly suppressed APAF-1 expression before and after IR.

Discussion

Ovarian torsion is the fifth most common gynecological emergency requiring immediate intervention. Since there are no specific symptoms and signs of ovarian torsion, delays in diagnosis and treatment are common[17]. Ischemia causes cell death, however during reperfusion, more tissue damage than ischemia alone in tissues occurs, which is known as ischemia-reperfusion injury

Table I. Descriptive statistics of TAS (μmol H2O2 Eq/L), TOS (μmol Trolox Eq/L), OSI (unitless) and AMH (ng/mL) values in groups.

	Sham	MC	IR	MC+IR	IR+MC	MC+IR+MC
Serum TAS	2.66 ± 0.21	2.57 ± 0.21	2.20 ± 0.13[a]	2.26 ± 0.16	2.37 ± 0.09	2.34 ± 0.11
Serum TOS	45.75 ± 9.80	67.66 ± 13.88	105.55 ± 22.88[a]	83.04 ± 15.12	56.47 ± 16.33[b]	57.12 ± 11.30[c]
OSI	17.40 ± 4.74	26.32 ± 4.97	47.76 ± 8.78[a]	36.52 ± 5.46	23.83 ± 7.16[b]	24.51 ± 5.43[c]
AMH	4.15 ± 0.68	3.83 ± 0.74	1.63 ± 0.48[a]	1.85 ± 0.68	2.31 ± 0.75	3.28 ± 0.98[c]

[a]Sham *vs*. IR, [b]IR *vs*. IR+MC, [c]IR *vs*. MC+IR+MC, letters show significance.

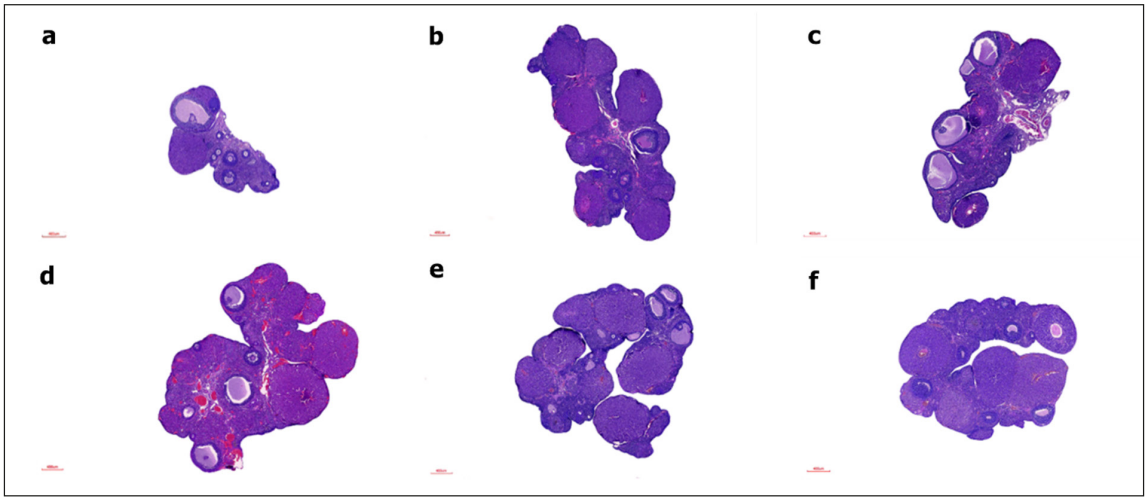

Figure 1. Total ovarian sections of (**a**) sham group; **b**, MC group; **c**, IR group; **d**, MC+IR group; **e**, IR+MC group; **f**, MC+IR+MC group. Hematoxylin Eosin, Bar: 400 µm.

(IRI)[18]. In addition to many histopathological changes, production of reactive oxygen species (ROS) increases in IRI[19].

Osmanağaoğlu et al[20] studied experimental ovarian IR measured TAS, TOS, and OSI values were 0.92±0.07 -0.84±0.40; 32.30±0.51 -41.42±16.71 and 3.52±0.86 -5.89±3.49 in the control group and IR group. The authors stated that the increase or decrease between the groups was statistically significant (p<0.05). In the histopathological scoring, mean values of follicular degeneration, vascular congestion,

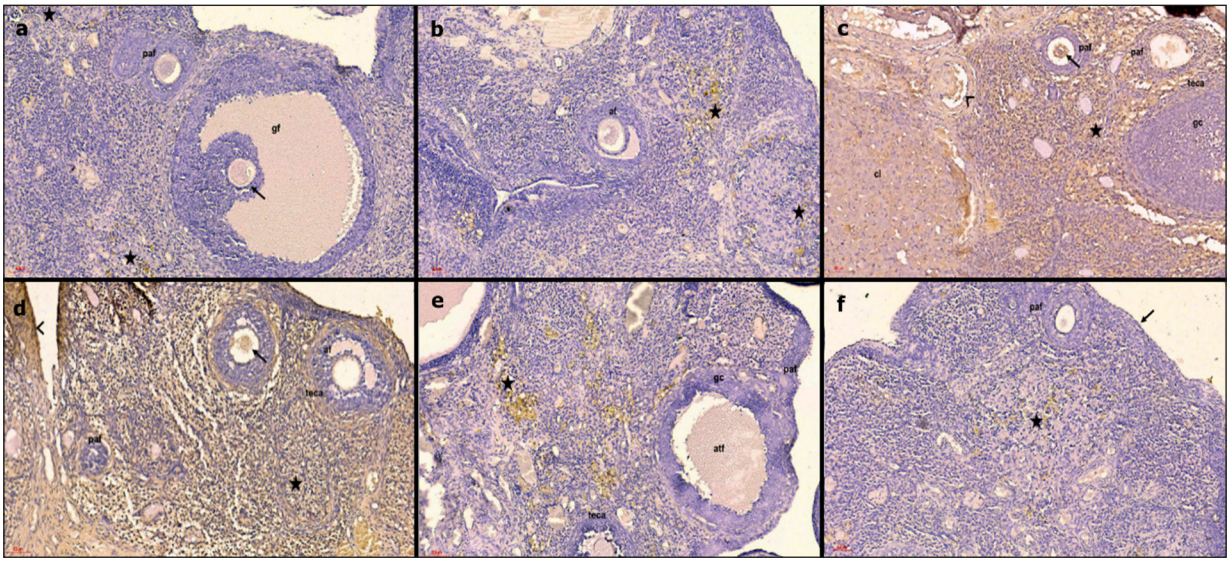

Figure 2. APAF-1 immunostaining of ovarian sections. **a**, Sham group, Negative APAF-1 expression in the pre antral (paf) and Graffian (gf) follicles and ovum (arrow), positive expression in a few stromal cells (asterisk). **b**, MC group, Positive APAF-1 expression (asterisk) in the cytoplasm of a few stromal cells, negative expression in antral (af) follicle. **c**, IR group, intense APAF-1 expression in preantral follicles (paf), ovum (arrow), theca cell layer (teca), granulosa cells (gc), vessels (arrowhead), corpus luteum (cl) and ovarian stroma (asterisk). **d**, MC+IR group, very high APAF-1 expression in the preantral (paf) and antral (af) follicles, ovum (arrow), theca cell layer (teca), ovarian stroma (asterisk) and germinal epithelium (arrowhead). **e**, IR+MC group; Negative APAF-1 expression in preantral (paf) and atretic (atf) follicles, theca (teca) and granulosa (gc) cell layers, positive expression in some stromal areas (asterisk). **f**, MC+IR+MC group; Negative APAF-1 reaction in preantral (paf) and germinal epithelium (arrow), mild positive reaction some stromal areas (star). APAF-1 immunostaining, Bar: 60 µm.

hemorrhage and inflammatory cell infiltration in the IR group were significantly higher than those in the control group ($p<0.001$). Similarly, Kalyoncu et al[16] recorded serum TAS, TOS, and OSI values and found TOS and OSI results were significantly high in the IR group. Regarding histopathological scoring of hemorrhage, congestion, degeneration, inflammation, all values were higher in the IR group compared to the control group. In another study[17] of ovarian IR, mean difference of TOS and OSI between control and IR groups was statistically significant ($p<0.001$). Compared to the control group, edema, vascular congestion, hemorrhage and inflammatory cell infiltration increased in the IR group ($p<0.001$).

In our study, serum TAS values were the lowest in the IR group. MC treatment increased the TAS value in the IRI applied groups compared to the IR group, but the increase was not statistically significant. TOS levels were significantly higher in the IR group compared to the sham and MC groups. After IRI, MC treatment decreased the high TOS level to a statistically significant level close to the value in the sham and MC groups. Similar to TOS results, a significant increase was observed in the IR group compared to the sham and MC groups, and a significant decrease was observed in the MC treatment groups compared to the IR group (Table I). Considering the TAS, TOS and OSI data, MC treatment increased the antioxidant level and decreased the oxidant level.

Histopathological results were shown in Figure 1. In ovarian sections of sham group (Figure 1a) and MC group (Figure 1b), ovarian histology was regular, folliculogenesis continued, corpus luteum was developed, and mesovarium were normal. In the IR group, degenerative follicles, vascular dilatation and congestion, increased mononuclear leukocyte infiltration, degeneration of granulosa cells and ovarian stroma were observed (Figure 1c). In the MC+IR group, similar histopathology of the IR group was observed, and the protective effect of MC treatment was weak (Figure 1d). In IR+MC (Figure 1e) and MC+IR+MC (Figure 1f) groups, folliculogenesis continued, dilatation and congestion in the vessels and connective tissue degeneration in the ovarian stroma decreased. The theca cell layer and corpus luteum were also observed. MC treatment after IRI effectively improved histological structural defects.

Anti-Müllerian Hormone (AMH), also known as Müllerian inhibitory substance (MIS), is a peptide hormone involved in the differentiation of sex-specific channels. The level of AMH decreases with age and is accepted as an indicator of ovarian reserve and fertility[18,19]. There are studies showing a relationship between decreased AMH level and IRI damage. Musayeva et al[20] measured AMH level as 2.09 ± 0.14 ng/mL in the sham group and 1.45 ± 0.21 ng/mL in the IR group in the ovarian IR (significant decrease, $p=0.035$). Eser et al[21] performed 2-hour ischemia and 2-hour reperfusion in ovary and measured the serum AMH. The authors found that AMH level 2.2±1.7 ng/mL in the sham group *vs.* 1.9±1.2 ng/mL in the IR group (significant decrease, $p=0.445$). In our study, a significant decrease was observed in the AMH level after IR compared to the sham ($p=0.001$). Administration of MC extract increased the AMH level in MC+IR and IR+MC groups. MC treatment before and after IR increased the AMH level and brought the ovarian reserve to a level close to that in sham group (Table I).

Apoptotic protease activating factor-1 encodes a cytoplasmic protein involved in apoptosis[22]. In the intrinsic signaling pathway of apoptosis, intracellular oxidative stress, endoplasmic reticulum stress, DNA damage as a result of IR cause the release of pro-apoptotic molecules, one of them is cytosolic APAF-1[23]. Studies[24] have shown that APAF-1 expression increases due to IRI. Zou et al[25] reported that cardiac IRI caused apoptosis in cardiomyocytes, and increased Apaf-1-cytochrome complex formation and the number of apoptotic cells and fragmented DNA. Kit et al[26] investigated APAF-1 expression in renal IRI and found that proapoptotic APAF-1 expression increased 11.2 times in IR group compared to the sham group ($p<0.005$). In the hepatic IR study performed by Bektas et al[27], the intensity of immune activity of APAF-1 in the sham group was 0.6±0.51 and 2.4±0.51 in the hepatic IR group (0: no expression, 3: intense). Baş et al[28] conducted ovarian IRI and reported that APAF-1 expression was weak in the control group but strong in the IR group with many apoptotic cells.

In our study, APAF-1 protein immune activity was shown in Figure 2. In ovarian sections of the sham group (Figure 2a) and MC group (Figure 2b), APAF-1 immune reaction was mostly negative. Compared to the sham and MC groups, a dramatic increase in APAF-1 expres-

sion was observed in the IR group (Figure 2c) and MC+IR group (Figure 2d). In the IR+MC (Figure 2e) and MC+IR+MC (Figure 2f) groups, negative APAF-1 expression was observed in the ovarian follicles.

MC treatment suppressed APAF-1 expression after IRI, yet the treatment was most effective when administered before and after IRI.

Limitations

Although effects of *Momordica charantia* were more effective after post IR injury, the conclusion was based on only illustrative results. Serum APAF-1 level measurement and quantitative techniques could be performed to support the hypothesis stronger.

Conclusions

In conclusion, administration of MC extract before IRI has low antioxidant and protective effects on ovarian tissues however the treatment is more effective after IRI by improving adverse effects of ovarian IRI and suppressing APAF-1 activity.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

Ethics approval was obtained by Dicle University Animal Experiments Local Ethics Committee with protocol number 2020/08.

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Authors' Contribution

FA and ÇÖ conceptualized and designed the experiments, performed the experimental protocols, and were equally contributor in writing the manuscript. All authors read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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