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Could Lycopene Protect Against Ischemia/Reperfusion Injury in the Uterus?

Likopen Uterustaki İskemi/Reperfüzyon Hasarına Karşı Koruyabilir mi?

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ABSTRACT

Introduction: Ischemia/reperfusion damage (IRD) is one of the most important factors in the success of organ transplantation. IRD plays a role in both acute and chronic rejection. This study aims to evaluate the effects of lycopene, which is known to be a powerful antioxidant, on experimental uterine IRD.

Methods: Twenty-four albino Wistar rats were included in the study and divided into three groups. No substances were administered the sham and ischemia/reperfusion groups. The ischemia/reperfusion and ischemia/reperfusion + lycopene groups were administered ischemia for 1 h and reperfusion for 2 h. The ischemia/reperfusion + lycopene group was administered 2.5 mg/kg lycopene reperfusion intraperitoneally half an hour before ischemia/reperfusion. Both uterine horns were extracted at the end of the procedure. Oxidative stress, inflammation, and apoptosis in the tissues were assessed.

Results: The malondialdehyde level, nuclear factor kappa-B immunoreactivity, and apoptotic cell number in the lycopene-administered group (ischemia/reperfusion + lycopene) were significantly decreased compared with those in the ischemia/reperfusion group ($p<0.05$).

Conclusion: The study found that lycopene reduced the effects of IRD on uterine tissue. Lycopene may positively affect the short- and long-term success of organ transplantation.

Keywords: Ischemia/reperfusion damage, oxidative stress, organ transplantation, lycopene

ÖZ

Amaç: İskemi/reperfüzyon hasarı (İRH) organ transplantasyonunun başarısının önündeki en önemli etkenlerden biridir. İRH hem akut hem de kronik rejeksiyonda rol oynamaktadır. Bu çalışmanın amacı güçlü bir antioksidan olduğu bilinen likopenin deneysel uterus İRH'de etkilerini değerlendirmektir.

Yöntemler: Çalışmaya alınan 24 Wistar albino rat 3 gruba ayrılmıştır. Sham ve iskemi/reperfüzyon grubuna hiçbir madde verilmemiştir. İskemi/reperfüzyon ve iskemi/reperfüzyon + likopen grubuna 1 saat iskemi ve 2 saat reperfüzyon uygulanmıştır. İskemi/reperfüzyon + likopen grubuna 2,5 mg/kg dozda likopen reperfüzyon yarım saat önce intraperitoneal olarak verilmiştir. İşlemin sonunda her iki uterin horn çıkarılmıştır. Dokularda oksidatif stres, enflamasyon ve apoptozis değerlendirilmiştir.

Bulgular: Likopen uygulanan grupta (iskemi/reperfüzyon + likopen) malondialdehit seviyesi, nükleer faktör kappa-B immünoreaktivitesi ve apoptotik hücre sayısı iskemi/reperfüzyon grubuna göre önemli ölçüde azalmıştır ($p<0,05$).

Sonuç: Likopenin İRH'nin uterin doku üzerindeki etkilerini azalttığı saptanmıştır. Likopen, organ naklinin kısa ve uzun vadeli başarısını olumlu yönde etkileyebilir.

Anahtar Kelimeler: İskemi/reperfüzyon hasarı, oksidatif stress, organ transplantasyonu, likopen

Introduction

Ischemia/reperfusion damage (IRD) is a process that starts with the reduction of perfusion in the tissue due to organ transplantation and acute myocardial infarction and then enters the main damaging period by ensuring reperfusion in the tissue. Although ensuring the perfusion in the hypoperfused tissue is the first step of treatment, preventing reperfusion damage that emerges due to reperfusion and affects organ function still poses a challenge for clinicians. One example of this situation is myocardial infarction. Interventions undertaken to ensure reperfusion in the early period decrease morbidity and mortality. Additionally,

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myocardial reperfusion affects infarction size through inflammation (1). These results lead scientists to examine pharmaceutical agents and methods that can reduce damage during the reperfusion process as much as possible.

Uterus transplantation is gaining increasing attention in the presence of unrecoverable uterine factor-induced infertility. After the first uterus transplantation was performed, the first live birth from a transplanted uterus was also achieved (2). One of the important obstacles to the success of the transplantation procedure is IRD. That IRD plays a role in both acute and chronic rejection is an accepted hypothesis. It has been shown during surgery that the treatment administered in the post-ischemia period may decrease acute rejection and increase the long-term success of transplantation (3). Various agents have been used to prevent IRD in numerous studies (4).

Nuclear factor kappa-B (NF-κB) is a family of pleiotropic transcription factors that was defined in 1986 and has various functions, such as embryonic development of tissues and the immune response (5). Regulation of NF-κB signaling is a complex process involving numerous genes and extracellular mediators, including free radicals. The effects of NF-κB on transplantation have been shown in experimental transplantation models (6,7).

Carotenoids are known to prevent oxidative damage in the cell (8). Lycopene (LYC) is one of the 600 carotenoids commonly found in nature (especially in tomato). LYC, which has anticancer, anti-proliferative, and neuroprotective properties, is also one of the most powerful antioxidants in the carotenoid family (9,10). It is used to prevent the negative effects of chemotherapeutic drugs on ovarian function (8). To our knowledge, there have been no studies examining the effect of LYC on uterine IRD. This study aimed to assess the effect of LYC on uterine IRD biochemically, immunohistochemically, and histologically.

Methods

Animals

Experimental animals were obtained from Saki Yenilli Experimental Animals Production and Research Laboratory. The number of subjects to be included in the study was determined with a 95% confidence interval and 0.9243 test power (PASS-11). In a One-Way analysis of variance study, eight samples were obtained from each of three groups whose means were to be compared. A total sample of 24 subjects using an F-test with a significance level of 0.05 was obtained 0.9243 power to detect differences between the means versus the alternative of equal means. The study used 24 Wistar albino female rats weighing 200-220 g. The rats were housed at 20 °C-22 °C, in 55%-65% humidity, and on a 12:12 h light/dark cycle until the day of the experiment. The study was conducted in compliance with the international guidelines (ARRIVE guidelines) and ethical rules after the approval of the Saki Yenilli Experimental Animals Production and Research Laboratory Local Ethics Committee was obtained (approval number: 04, date: 24.02.2020). Patient approval has not been obtained as it is performed on animals.

Experimental Groups

Rats were dove into three groups: Ischemia/reperfusion (IR, n=8), ischemia/reperfusion + 2.5 mm/kg bw lycopene (LIR, n=8), and sham (n=8). All rats were synchronized at the diestrus phase of the estrous cycle in compliance with the definition provided by Marcondes et al. (11).

Experimental Procedure

Rats were anesthetized by intraperitoneal injection of a xylazine hydrochloride 7 mg/kg (Bayer, Turkey) and ketamine hydrochloride 50 mg/kg (Eczacıbaşı, Turkey) combination. The rats were held in a supine position, and the abdominal region was wiped with antiseptic solution after shaving. The abdomen was entered through a 2-3 cm vertical incision made in this area. The abdomen was opened and closed without performing uterine ischemia in the sham group. Uterine ischemia was formed using the microvascular bulldog clamps according to Sahin et al. (4). The duration of ischemia was set as 1 hour, and the LIR group was administered LYC intraperitoneally 0.5 h before reperfusion. Following completion of the planned reperfusion duration (2 h), the rats were sacrificed under anesthesia by the decapitation method, and both uterine horns were extracted. The left uterine horn was removed for histology and immunohistochemical evaluation. The right uterine horn was maintained at -80 °C for biochemical evaluation.

Histological Analysis

The extracted uterine tissues at the end of the experiment were fixed in 10% formaldehyde solution. After the fixation process, the tissues were dehydrated by passage through a graded alcohol series (50%, 70%, 80%, 96%, and 100%). The tissues were cleared with xylene and embedded in paraffin. Five-micron-thick sections taken from paraffin blocks were stained with hematoxylin-eosin (H&E) and fixed to slides with mounting medium (Entellan®, Merck). The prepared slides were examined under an Olympus BX53 light microscope.

Immunohistochemical Analysis

The immunoreactivity of the NF-κB protein in uterine tissues in the rat models on which IR performed was determined by the Avidin-Biotin peroxidase method. In brief, citrate buffer was used to open the epitopes after deparaffinization of the sections (5 µm) (pH 6.0). Then, the slides were immersed in 3% hydrogen peroxide solution in methanol to prevent endogenous peroxidase activity. Ultra V block solution was used to prevent non-specific staining. Then, the sections were incubated with primary antibodies at 4 °C overnight. Biotinylated secondary streptavidin-horseradish peroxidase and 3,3′-Diaminobenzidine chromogens were applied, respectively, and then the sections were reverse stained with Gill Hematoxylin. The sections were dehydrated in a graded alcohol series and fixed to slides with Entellan mounting medium. The sections were examined under an Olympus BX53 light microscope. The immunoreactivity levels were quantified using the Image J program. Ten different areas were evaluated for every slide.

Biochemical Analysis

Tissue samples obtained from rats were used for biochemical analysis. Rat malondialdehyde (MDA) was quantified in uterine tissue using a commercial kit (cat. no: 201-11-0157, Sunred Bio) according to the manufacturer's protocol, and the concentration was measured at 450 nm with an ELISA plate reader. The results are presented as nmol/mL of MDA.

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end Labeling

Apoptotic cells in the sections obtained from the subjects were detected using the In Situ Cell Detection Apoptosis Fluorescein Kit (Roche). Staining was performed according to the manufacturer's instructions. Five-micron-thick uterus sections were deparaffinized and rehydrated then washed with PBS twice for 5 min each time. Afterwards, they were kept in a microwave oven in 0.01 M 5% sodium citrate buffer for 5 min at 350 W for antigen recovery then left to cool at room temperature for 10 min. Tissues were washed twice for 5 min each time with PBS then placed in a humidity chamber at 37 °C with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction mixture included in the kit and incubated in the incubator for 60 min. Opposite staining was performed on the tissues that were washed two times for five min each time with PBS using 4',6-diamidino-2-phenylindole. The tissues were covered with glycerol sealing solution and scanned at wavelength of 450-500 nm in an Olympus BX53 model florescent microscope. Apoptotic cells in 50 different areas under 200x objective magnification were counted for calculation of the apoptotic index by using the Image J program.

Statistical Analysis

The SPSS 22 statistical software program was used for the statistical analyses (SPSS Inc., Chicago, IL). The results are presented as median (IQR). The normality of the data distribution was evaluated by visual (histogram) and analytical (Kolmogorov-Smirnov test) methods. Intergroup comparisons were carried out with the One-Way Kruskal-Wallis test. The post-hoc Tamhane test was used for binary comparisons. Results were considered significant at $p<0.05$.

Results

Histochemical Results

Figure 1 shows the histopathological evaluation results. Accordingly, normal histological structures were observed in uterine tissues of the sham (Figure 1A) groups. The study observed that the lumen epithelium and glandular epithelium cells were vacuolized in the uterus section of the ischemia/perfusion (Figure 1B) group. The study also detected intensive neutrophil infiltration in endometrial areas. Vacuolization in the lumen epithelium and glandular epithelium in the IR + LYC group increased, and neutrophil infiltration decreased (Figure 1C).

Immunohistochemical Results

Figure 2A shows the NF-κB immunoreactivity in the uterine tissue. Accordingly, NF-κB expression increased significantly in the uterine tissue, which was only administered ischemia perfusion (IR) (Figure 2B), compared with all other experimental groups ($p<0.05$). NF-κB expression decreased significantly in uterine tissues that were administered 2.5 mg/kg LYC (Figure 2C), compared with the IR group ($p<0.05$).

Biochemical Results

The evaluation of the MDA results showed that there was a significant increase in the IR group compared with the control group. There was a significant decrease in the LYC group compared with the IR group (Table 1).

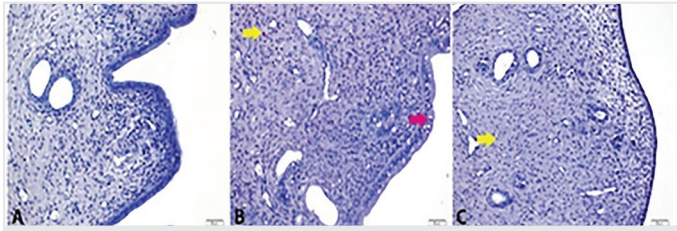

Figure 1. Hematoxylin-eosin staining images of uterine tissues of Sham A), ischemia/reperfusion B), and 2.5 mg/kg lycopene C) experimental groups. The pink arrow shows the vacuolization in lumen epithelium and glandular epithelium cells, and the yellow arrow shows a neutrophil cell

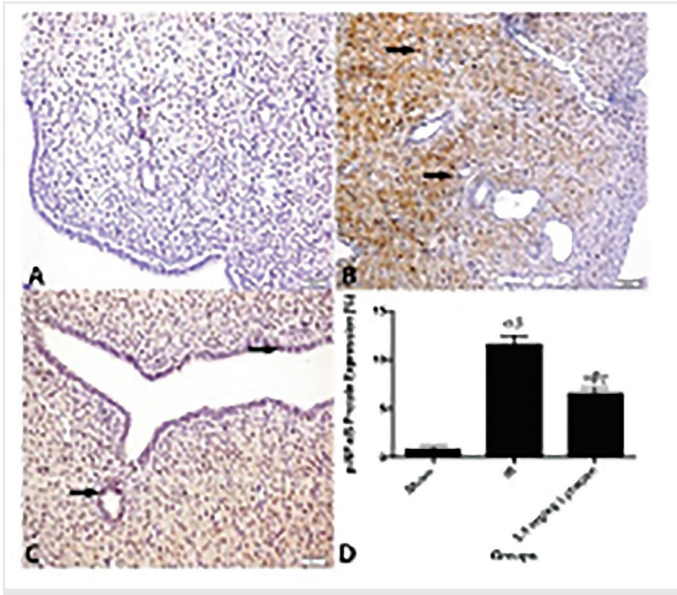

Figure 2. Nuclear factor kappa-B (NF-κB) immune staining in the uteruses of Sham A), ischemia/reperfusion (IR) B), and 2.5 mg/kg lycopene C) experimental groups. The black arrow shows the immunoreactive areas. NF-κB immunoreactivity data showed in the histogram graphic were expressed as mean ± standard deviation (a $p<0.05$ sham group; b $p<0.05$ IR group; g $p<0.05$ 2.5 mg/kg lycopene group)

Table 1. MDA and TUNEL results in the uterine tissue

Groups	Sham	IR	LIR	p
MDA (nmol/mL)	0.74 (0.11)[a]	2.42 (0.45)[b]	0.86 (0.11)[a]	<0.001
TUNEL (+) cell	1 (1)[a]	7 (3.5)[b]	1 (2)[a]	<0.001

The data were presented as median (IQR). The significance level was $p<0.05$. No significant differences were found between the groups with the same letters (a, b).
MDA: Malondialdehyde, TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, IR: ischemia/reperfusion, LIR: lycopene

TUNEL Results

Table 1 and Figure 3 show the evaluation results of the apoptotic cells in the uterine tissue. Accordingly, the number of apoptotic cells significantly increased in the sham group compared with the IR group while 2.5 mg/kg LYC administration significantly decreased the increased number of apoptotic cells after IR.

Discussion

Oxidative stress-induced reperfusion damage increases acute and chronic rejection. Reducing IRD may increase the success of organ transplantation (3). This study examined the corrective effect of LYC on uterine IRD. MDA levels, NF-κB immunoreactivity, and the apoptotic index in the LIR group were significantly lower than those in the IR group. This experimental study revealed that LYC had positive effects on uterine IRD.

Oxidative stress, which causes the formation of reactive oxygen radicals (ROS), is the main outcome of IRD. Along with tissue hypoxia, dysfunction emerges in the electron transport chain in mitochondria. Decreased adenosine triphosphate (ATP) production in mitochondria increases anaerobic metabolism and impairment in the sodium-potassium pump. A vicious cycle starts during this period. Anaerobic metabolism causes decreased ATP and antioxidant agent production (12). Intracellular osmolarity increases, and pH decreases with the deterioration in the ion channels. The generation of ROS, which causes oxidative stress, increases due to low antioxidant defense in ischemic cells with reperfusion. Inflammatory processes and oxidative stress may cause cell damage with the cytokine production they induce (13). IRD is one of the factors that affect the short- and long-term success of organ transplantation (3). The severity of organ damage varies with the warm ischemia time, which is defined as the amount of time spent between extracting the organ from the body and washing it with hypothermic preservatives (14). A uterine transplantation study conducted on rats showed that a warm ischemia time longer than 5 h decreases the success of transplantation (14). Kisu et al. (15) revealed that the uterus might be more resistant to ischemia than other organs.

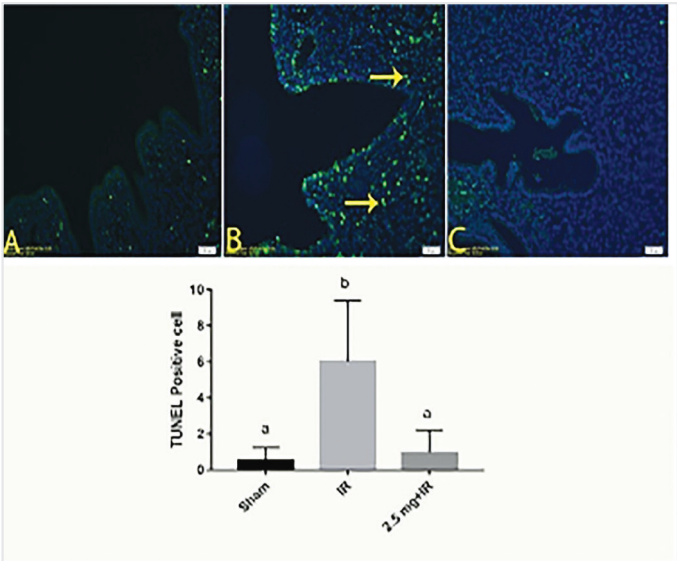

Figure 3. A) Sham group, B) IR group, C) 2.5 mg + IR group. The yellow arrow shows the apoptotic cells, x200

Previous studies used various substances such as pharmacologic agents in uterine IRD (4). LYC antioxidant, a natural carotenoid, is often used in experimental studies due to its antiapoptotic and anti-inflammatory prosperities (16). Another reason for using LYC frequently is that it is not toxic (17). Bayramoglu et al. (16) reported that LYC had a protective effect on liver IRD. Lei et al. (18) found that LYC had a neuroprotective and antiapoptotic effect on brain ischemia by increasing nuclear factor erythroid 2-related factor and heme oxygenase-1 expression levels. Premature ovarian failure may emerge as an undesirable effect of cancer treatment at the reproductive age. A study examining the effects of chemotherapeutics on the ovary determined that LYC positively affected fertility by reducing ovarian failure (19). However, there are no studies that examine the effect of LYC on uterine IRD.

Xanthine oxidoreductases consisting of xanthine dehydrogenase and xanthine oxidase are active in the process that breaks purine up to uric acid. Although various enzymes play a role in purine metabolism, xanthine oxidase has an important role in the formation of ROS. Toxic substances such as MDA form with lipid peroxidation due to increased ROS (20). The MDA level is one of the best indicators of oxidative stress. Ischemia-reperfusion studies showed that the MDA levels were high (4). The present study found that the MDA was significantly different between the IRG and LIRG groups, in line with the literature. The study showed that LYC had a positive effect on oxidative stress in the uterus.

ROS leads to activation of the cytokine cascade and increased expression of adhesion proteins (21). NF-κB is one of the redox-sensitive proinflammatory transcription factors. The effects of oxidative stress on the NF-κB pathway were assessed in different studies. Tumor necrosis factor alpha, lipopolysaccharide, and interleukin-1 were found to be NF-κB pathway activators (22). A subsequent study in human T cells showed that very low levels of hydrogen peroxide can activate the NF-κB pathway (23). However, the latest studies have indicated that hydrogen peroxide may be a modulator rather than an activator (24). This turns NF-κB into the target molecule in oxidative stress studies. It was found that NF-κB inhibition with JSH-23 increased oxidative stress in the hearts of rats with hereditary hypertriglyceridemia (25). On the other hand, cannabinoid-2 receptor activation with JWH-133 was found to significantly reduce the number of NF-κB-positive cells in kidney tissue and thus reduced kidney damage (26). The present study showed that LYC significantly decreased NF-κB immunoreactivity. This decrease in immunoreactivity may be due to the decreasing effect of LYC on IR.

IRD-induced ROS plays a role in various processes, such as cell proliferation and apoptosis. Apoptosis, autophagy, necrosis, and necroptosis may occur in the cell with prolonged IRD (12). Numerous studies showed that apoptosis has an important role in IRD (27). The majority of cell deaths after ischemia are due to apoptosis (28). Therefore, the opinion that "prevention of apoptosis reduces IRD" is coherent. A study examining the effect of procyanidin on myocardial IRD revealed that procyanidin reduced myocardial IRD by inhibiting the apoptotic pathways (29). Apoptosis is assessed using techniques based

on morphologic changes, cytoplasmic changes, DNA fragmentation, and membrane changes in the cell. The TUNEL technique assesses apoptosis through DNA fragmentation. There was a significant difference between the groups in terms of the number of TUNEL-positive cells in this study. This result shows the antiapoptotic propensity of LYC in uterine IRD.

Conclusion

LYC, a natural carotenoid, can be an alternative for reducing organ rejection, which is an important problem in organ transplantation. LYC reduced oxidative stress, inflammation and apoptosis in uterine IRD, even in low dosages, such as 2.5 mg/kg. Due to the wide availability of LYC in nature, the required amount can be provided with a simple diet change. Moreover, its non-toxicity is an important advantage.

Ethics Committee Approval: The study was conducted in compliance with the international guidelines (ARRIVE guidelines) and ethical rules after the approval of the Saki Yenilli Experimental Animals Production and Research Laboratory Local Ethics Committee was obtained (approval number: 04, date: 24.02.2020).

Informed Consent: Patient approval has not been obtained as it is performed on animals.

Peer-review: Externally peer-reviewed.

Authorship Contributions: Surgical and Medical Practices - T.O., M.D.Ç.; Concept - T.O., Z.D.; Design - T.O., M.D.Ç., C.T., M.M.A.; Data Collection or Processing - Z.D., E.K.; Analysis or Interpretation - T.O., Z.D., M.D.Ç., C.T., E.K., M.M.A.; Literature Search - T.O., M.D.Ç.; Writing - T.O., Z.D.

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