The Effect of Selenium on Ischemia-Reperfusion Injury: An Experimental Study on a Transverse Rectus Abdominis Musculocutaneous Flap Model

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Background: The aim of this study is to investigate effects of selenium and enlighten the possible mechanism of action in a rat transverse musculocutaneous flap model following ischemia-reperfusion injury.

Materials and Methods: In this study, an experimental model, which mimicked free tissue transfer, was applied. Twenty-four male Wistar Albino rats were divided into a control group (N = 12), and a selenium treated group (N = 12). A superiorly based transverse rectus abdominis musculocutaneous (TRAM) flap was elevated and an ischemic insult for 4 hours was given. In selenium treated group (Group 2), sodium selenite (0.625 mg/kg) was injected intraperitoneally (i.p), 2 hours before the induction of ischemia. Six rats from each group were sacrificed at 24 hours after the operation and malonyldialdehyde (MDA), nitric oxide (NO), and glutathione (GSH) levels were measured biochemically, whereas the intensity of neutrophil infiltration was evaluated. For the rest of the rats in Group 2, sodium selenite was injected at the same dose everyday to the postoperative 10th day, in which the remaining 6 rats from each group were sacrificed. On postoperative 10th day, flap viability was assessed along with the evaluation of intensity of neovascularization.

Results: In Group 1, MDA levels were higher significantly ($P < 0.05$) when compared with Group 2. No statistical difference, however, was found for NO ($P > 0.05$), and GSH ($P > 0.05$) levels among Group 1 and 2. Neutrophil infiltration was more intense in Group 1, when compared with Group 2 whereas neovascularization was more abundant in samples of Group 2. Group 2 shows higher average flap surface areas when compared with Group 1 ($P < 0.05$).

Discussion: The results of this study demonstrated the preventive effect of selenium against ischemia-reperfusion injury by reducing tissue necrosis in muscle flaps possibly by decreasing MDA, increasing neovascularization, and decreasing neutrophil infiltration, thus suppressing inflammation.

Key Words: Glutathione, malonyldialdehyde, musculocutaneous flap, nitric oxide, selenium

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Selenium is found in the structure of selenoproteins, such as thioredoxin reductase and glutathione peroxidase,[1] however, it cannot be produced in human body and is ingested through diet. Five different isoenzymes of glutathione peroxidase (GPx) exist in humans and are tissue specific.[2,3] Glutathione peroxidase enzymes catalyze reduction of hydrogen peroxides and organic hydroperoxides to protect cells from oxidative stress.[3] Selenocisteine, glutamine, and tryptophan forms a catalytic center in the structure of glutathione peroxidase, which is essential for the catalytic function of GPx enzymes.[4] Selenium-containing enzymes play important roles in protection against peroxidation processes.

Ischemia and reperfusion of tissues normally occur during replantations, solid organ transplantations, and free flap transfers. Acute interruption of blood supply to the affected tissue, however, followed by reperfusion for prolonged periods result in ischemia-reperfusion injury. During the ischemic period, oxygen, glucose, and adenosine triphosphate levels decrease abruptly, whereas carbon dioxide and lactic acid levels increase because of the activation of anaerobic metabolism. During ischemia period, chemotactic signals, which are produced by thrombocytes, endothelial cells, and other leucocytes, direct neutrophils to the inflammatory region.[5] Reactive oxygen radicals and their metabolites also cause the formation of chemotactic stimulus.[6] During the reperfusion of ischemic tissues inflammatory mediators are produced and this cause the rolling, adhesion, and migration of leucocytes.[7] Neutrophils cause endothelial injury, which in turn result in disruption of microvascular integrity, edema, thrombosis, and tissue necrosis.[8]

Until now, several studies have shown beneficial effects of selenium in ischemia-reperfusion injury of various organs.[9,10,11,12,13,14] The aim of this study, is to investigate the effects of selenium on transverse rectus abdominis musculocutaneous (TRAM) flaps of rats and discuss the possible mechanisms of action following ischemia-reperfusion injury.

MATERIALS AND METHODS

All experiments were performed with the approval of the Institutional Animal Care and Use Committee of Inonu University, School of Medicine (Approval No: 2009/18). Twenty-four male Wistar Albino rats, aged 7 to 8 weeks and weighing between 170 to 220 g, were kept 1 per cage with free access to standard hamster food and water at a laboratory with a room temperature between 18 to 20 °C,

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in humidity (60, ±5%) controlled rooms in which a 12:12 hours of light:dark cycle was maintained. All procedures were performed in accordance with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals.

The animals were divided into 2 groups which consisted of 12 rats each. Group 1 (Group I/R) was the control group and Group 2 (Group I/R + Se) was treated with selenium (sodium selenite (Na_2O_3Se), ABCR GmbH & Co.KG, Karlsruhe, Germany) intraperitoneally.

SURGICAL TECHNIQUE

The rats were anesthetized with intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). No antioxidant agent was administered in Group 1. In Group 2, 2 hours before the operation, sodium selenite was injected (0.625 mg/kg) through intraperitoneal route (i.p). All the operative procedures are performed aseptically, after preparing the abdomen. Left-sided TRAM flap (pedicle: single superior epigastric artery and vein) with a 5 × 4 cm skin component was planned (Fig. 1, left). Flap was elevated (Fig. 1, middle). Skeletonization of flap pedicle (left superior epigastric artery and vein) is performed after visualization of pedicle by transillumination and, a microvascular clamp is applied to the pedicle to achieve an ischemic insult for 4 hours (Fig. 1, right). Suture ligation of contralateral superior epigastric vessels and inferior epigastric vessels of both sides are performed. After 4 hours of ischemic insult, the microvascular clamp is removed and, the flap is thus reperfused. Flaps are repositioned and sutured. After wound closure, gentamicin (3 mg/kg) was injected through i.p route. Six rats from each group (half of the rats from each group) were sacrificed at 24 hours after the operation to measure nitric oxide activity, malonyldialdehyde activity, and glutathione levels in addition to the evaluation for severity of neutrophil infiltration. The remaining 6 rats from Group 2, which were left to survive for 10 days after the operation received sodium selenite (dose: 0.625 mg/kg, i.p) each day. At day 10, the remaining 6 rats from each group were sacrificed and gross measurement of flap survival and the intensity of neovascularization was performed. All rats, which will survive for 10 days in both groups, were injected with gentamicin (3 mg/kg) each day, until they are sacrificed.

HISTOLOGIC EVALUATION

The specimens were obtained from Group 1 and Group 2. The semiquantitative analysis of the samples was carried out by the same pathologist who was blinded to the samples. The specimens were obtained from the distal portions of flaps, including muscle and skin. Specimens are fixed in 10% of formalin solution, which then were paraffin embedded and slices of 4 to 5μ of thickness were obtained, which were then stained with hematoxylin and eosin. The specimens obtained at postoperative 24 hours were evaluated for neutrophil infiltration intensity and, the specimens obtained at postoperative 10 days were evaluated for the intensity of neovascularization under light microscope and were scored by the same blinded pathologist. The scoring system is composed of following items: (−) few, (+) mild, (++) moderate, and (+++) severe.

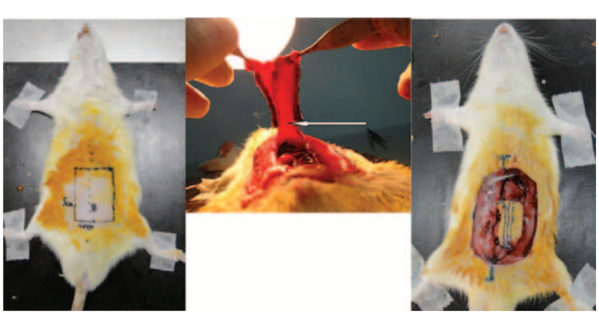

FIGURE 1. Planning of a single superior epigastric artery and vein pedicled, left-sided transverse rectus abdominis musculocutaneous flap with a 5 × 4 cm skin component (left). Flap is elevated and the arrow shows superior epigastric artery and vein, which became apparent via transillumination (middle). After the skeletonization of flap pedicle, microvascular clamp is applied to the left superior epigastric artery and vein to achieve an ischemic insult for 4 hours (right).

TABLE 1. The Effects of Selenium Administration on Tissue Enzymes in Rats With TRAM Flap Elevation

Groups	Malonyldialdehyde (nmol/g)	Nitric Oxide (nmol/g)	Reduced Glutathione (nmol/g)
Group 1	9233 ± 4112	37,233 ± 5338	190,650 ± 12,957
Group 2	58,667 ± 1051	40,833 ± 5443	1865 ± 11,456

TRAM, transverse rectus abdominis musculocutaneous.

BIOCHEMICAL EVALUATION

To determine the levels of nitric oxide (NO), glutathione (GSH), and malonyldialdehyde (MDA), minimum 100 mg of tissue is obtained for each parameter, from the distal part of flaps. The tissues, has been obtained from both groups after sacrification of 6 rats in each group at postoperative 24 hours. The specimens obtained were weighed and phosphate tampon was added to form 10% of homogenate. Then it is homogenized inside the ice at 12,000 rpm/min for 1 to 2 minutes. Malonyldialdehyde analysis of the obtained tissue homogenates was performed according to the method described by Ohkawa et al.[15] The rest of the homogenates were centrifuged at 5000 rpm and, 4°C for 30 minutes. Supernatant samples were used in the analysis of MDA, NO, and GSH.

10% of trichloroacetic acid (TCA) solution is added to the supernatant samples and stirred, then centrifuged at 4°C and 3000 rpm for 20 minutes, which made proteins precipitate. Glutathione analysis of supernatant samples, which are light colored and have no proteins was performed according to Ellman method.[16]

ASSESSMENT OF FLAP VIABILITY

At 10 days after the operation last 6 rats from each group are sacrificed. Viability of flaps is determined based on color and eschar formation. The viable areas and nonviable areas are plotted on a transparent plastic paper so that a three-dimensional surface area is transformed to a two-dimensional one to make accurate calculation of viable and total surface areas. The viable portion and total flap surface area of each flap is calculated using Auto-CAD (Auto-CAD 2009, Autodesk, San Rafael, CA), a commercially available program. The viable surface areas are proportioned to total flap surface areas to calculate the percentage of viable flaps.

STATISTICAL ANALYSIS

All data were analyzed by using the SPSS 13.0 software package (SPSS, Inc., Chicago, IL). Mean values, standard deviation,

TABLE 2. Intensity of Neutrophil Infiltration in Flap at 24 hours After the Operation

Rat samples	Group 1	Group 2
1	++	++
2	+++	++
3	+++	+
4	++	+
5	++	+++
6	++	+

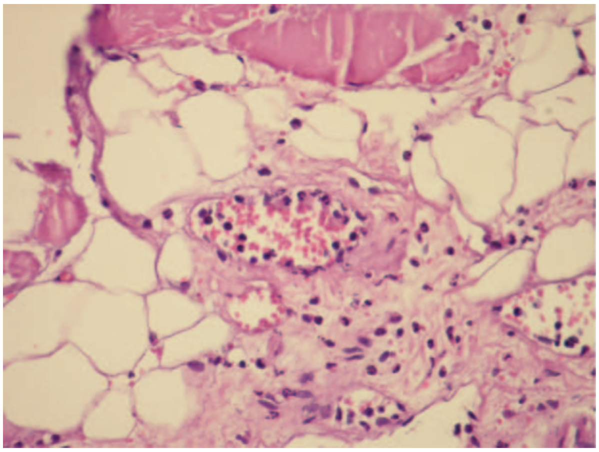

FIGURE 2. Microscopic findings at postoperative 24 hours. Increased perivascular infiltration of neutrophils and adhesion on endothelial walls (indicated by arrow) are seen in the control group (hematoxylin and eosin, ×40).

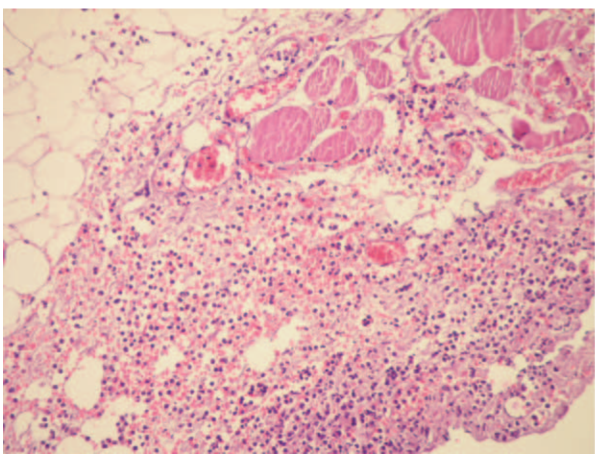

FIGURE 3. Severe neutrophil infiltration inside the fatty tissue and between muscle fibers (indicated by arrow) is seen at postoperative 24 hours in Group 1 (hematoxylin and eosin, ×20).

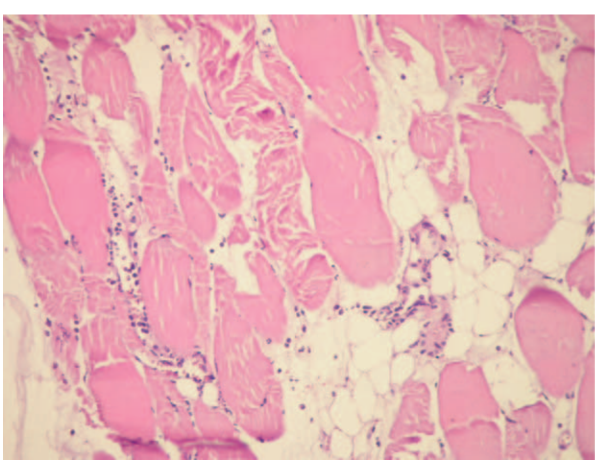

FIGURE 4. Mild neutrophil infiltration between muscle fibers (indicated by arrow) is seen at postoperative 24 hours in Group 2 (hematoxylin and eosin, ×20).

TABLE 3. Intensity of Neovascularization in Flap 10 Days After the Operation

Rat Samples	Group 1	Group 2
1	+	+
2	+	+
3	−	++
4	−	++
5	++	++
6	+++	++

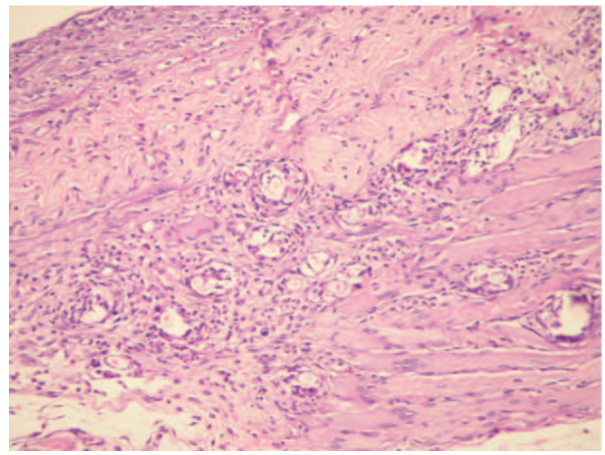

FIGURE 5. Intensive neovascularization (indicated by arrows) is seen in Group 2 at postoperative day 10 (hematoxylin and eosin, ×20).

minimum, and maximum values are calculated. For the comparison of 2 groups Mann-Whitney test is used for variables, which do not show normal distribution and, *t*-test is used for variables of normal distribution. To identify the homogeneity of variables, Levene statistics is used.

RESULTS

Biochemical Assays

Malonyldialdehyde levels were significantly higher in Group 1 when compared with Group 2 ($P < 0.05$), the selenium treated group (Table 1). Nitric oxide levels ($P > 0.05$) and reduced GSH

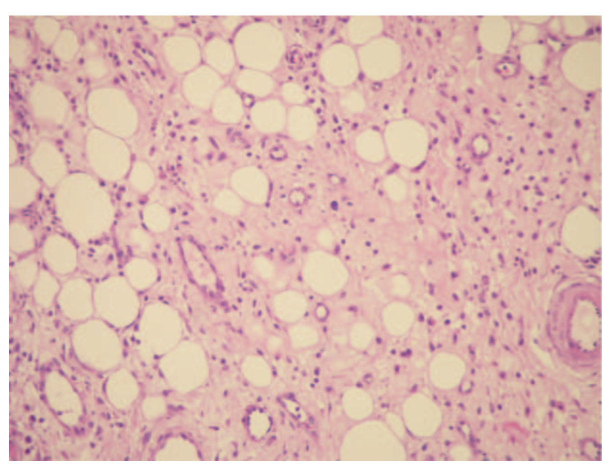

FIGURE 6. Mild neovascularization (indicated by arrow) is seen in Group 1, at postoperative day 10 (hematoxylin and eosin, ×20).

levels ($P > 0.05$) did not show statistical significance among the 2 groups.

Histologic Evaluation Results

Table 2 shows intensity of neutrophil infiltration at 24 hours postoperatively among Group 1 and Group 2 (Table 2). More abundant neutrophil infiltration is observed in Group 1 (Figs. 2-4).

Table 3 shows intensity of neovascularization at 10 days postoperatively among the 2 groups (Table 3). Group 2 exhibits more intensive neovascularization when compared with Group 1 (Figs. 5-6).

Flap Survival Rate

Group 2 shows higher viable flap surface areas when compared with Group 1 ($P < 0.05$) (Fig. 7). Table 4 shows average flap survival rates of each group (Table 4).

TABLE 4. Average Flap Survival Rates of Group 1 and Group 2 Are Shown

Groups	Average Flap Survival Rates
Group 1	$24{,}925 \pm 15{,}194$
Group 2	$83{,}305 \pm 8642$

DISCUSSION

Ischemia and reperfusion occurs during organ transplantations, replantations, and free flap transfers. Primary ischemia starts with clamping the vascular pedicle and, ends with the maintenance of reperfusion.[17] Restoration blood flow is of vital importance for the survival of ischemic tissues, however, reperfusion may even further harm ischemic tissues known as ischemia-reperfusion injury[18] because of reactive oxygen radicals, such as superoxide, hydrogen peroxide, and hydroxyl radicals, which are produced during reperfusion.[1] Reactive oxygen radicals result in vasoconstriction, increased capillary permeability, and edema in the endothelial cells thus, impairing microcirculation.[17] Selenium is found in the structure of selenoproteins, such as glutathione peroxidase (GPx), thioredoxin reductase,[1] which are effective in reduction of hydrogen peroxides and organic hydroperoxides,[3] and have antioxidant capacity. Five different GPx isoenzymes, which show tissue-specific expression and include selenosisteine, exist in humans.[2,3] It is reported that the integrity of triple catalytic center formed by selenocisteine, glutamine, and tryptophan residues is essential for the catalytic function of GPx enzymes.[4] Selenoprotein P, the major selenoprotein in human plasma,[19] exhibits phospholipid hydroperoxide glutathione peroxidase activity in extracellular medium,[20] and is also protective against the effects of peroxynitrite and the oxidation and nitration as a result of peroxynitrite.[21] Selenoprotein W is another selenoprotein, which shows antioxidant capacity.[22] Selenium may be accounted as an important substrate because it is present in the structure of so many antioxidant proteins. Until now, several studies have shown beneficial effects of selenium administration in ischemia-reperfusion injury of cerebral tissues,[13,14] myocardium,[9,10] and intestines.[11]

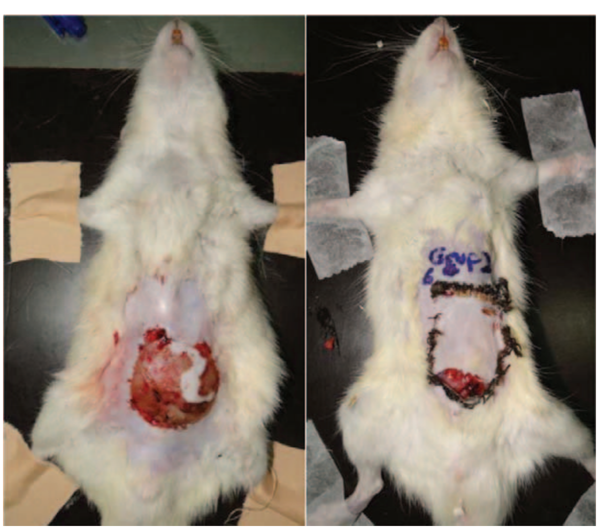

FIGURE 7. An example of partial flap losses in control group (Group 1), evaluated on postoperative day 10 (left). An example of partial flap losses in the selenium treated group (Group 2), evaluated on postoperative day 10.

In case of selenium deficiency, selenoprotein P, shows antioxidant activity, however, if selenium support is adequate, cytosolic GPx is protective against oxidative stress.[23] Lipids are present abundantly in the structure of cell membranes and MDA is the quantitative marker of lipid peroxidation. Malonyldialdehyde levels in Group 2 are found statistically lower than Group 1. This result may be secondary to the effects of selenoproteins and glutathione peroxidase isoenzymes.

Inflammatory mediators, which are produced during I-R injury, cause the rolling, adhesion, and migration of leucocytes,[7] which in turn cause endothelial injury thus, result in disruption of microvascular integrity, edema, thrombosis, and tissue necrosis.[8] Tissue specimens obtained at 24 hours, revealed more abundant neutrophil infiltration in Group 1 when compared with Group 2. Increased neutrophil infiltration is related with increased malonyldialdehyde and increased myeloperoxidase (MPO) levels.[24] Decreased neutrophil infiltration in selenium treated group diminishes lipid peroxidation and thus MDA levels. Selenium treatment diminishes neutrophil infiltration and lipid peroxidation in muscle flaps.

Glutathione peroxidase catalyses reduction of lipid and organic hydroperoxides.[25] This selenium, including enzyme uses sulfhydryl groups of GSH as hydrogen donor thus, forming oxidized disulfide forms of GSH (GSSG) (1). In our study, no statistical difference was obtained between two groups among GSH levels. During ischemia-reperfusion injury, GSH is continuously oxidized and reduced to remove hydrogen peroxide (H_2O_2) from the environment. This shows that GSH is not an end product. This is the reason why we think that GSH and/or GSSG may not be used for the assessment of effectiveness of any agent in ischemia-reperfusion injury. Instead of it, the increase in the activity of GPx may be used as a more valuable data for this purpose.

Meldrum et al[26] reported that NO exhibits both beneficial and harmful effects during ischemia-reperfusion injury. At the beginning of ischemia, conductive nitric oxide synthase (cNOS) produce NO through L-arginine, which depletes L-arginine locally.[27] When L-arginine levels are lowered, O_2^- is produced instead of NO.[27] When endothelium is injured in I-R injury inductive nitric oxide synthase (iNOS) and endothelin 1 is expressed.[28] This in turn increase total endogenous plasma NO levels but threatens flap viability.[28] Nitric oxide production through iNOS exhibits harmful effects on flap viability.[28] In this study, NO levels are measured at 24 hours after the operation and, no statistically significant difference could be obtained between the 2 groups. Conductive nitric oxide synthase and iNOS levels, however, are not measured. If cNOS and iNOS levels were measured in this study, we could have a better understanding about the clinical effects of selenium on NO production.

Ischemia-reperfusion injury results in tissue injury and tissue necrosis. In this study, we calculated viable area proportions of flaps using Auto CAD 2009 program. The proportions of viable surface area of flaps to total flap surface area of Group 2 is statistically higher when compared with Group 1. This result shows that selenium is an effective agent in reducing tissue necrosis of muscle flaps following ischemia-reperfusion injury.

Neovascularization occurs as a result of an effort of the host to meet increased tissue demands. The results of this study show that neovascularization was more intense in Group 2 when compared with Group 1. Selenium increases neovascularization in muscle flaps.

The results of this study demonstrated the preventive effect of selenium against ischemia-reperfusion injury by decreasing neutrophil infiltration, by lipid peroxidation during the critical postoperative 24 hours. Selenium increases neovascularization and is effective in reducing tissue necrosis in muscle flaps.

Furthermore studies must be performed for more detailed understanding of the mechanism of action of selenium in ischemia-reperfusion injury of musculocutaneous flaps and for possible future clinical use.

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