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EFFECTS OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) ON REMOTE ORGANS IN AN EXPERIMENTAL MODEL OF INTESTINAL ISCHEMIC REPERFUSION INJURY

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ABSTRACT

Ischemic reperfusion injury (IRI) is a dual process with local damage due to hypoxia during ischemia and additional local and systemic injury due to inflammation and oxidative damage during reperfusion. Vascular endothelial growth factor (VEGF) is a protein known to stimulate vasculogenesis and angiogenesis. It is also accepted to alleviate the effects of IRI as it has antioxidant and vasodilator properties. The aim of this study is to evaluate the remote organ effects of VEGF in an experimental model of mesenteric ischemia and reperfusion.

After obtaining Animals Ethic Committee approval, 24 male Wistar Albino rats were assigned to 4 groups. All animals underwent a midline laparotomy and dissection of mesenteric artery. Sham group received no further intervention (Gr S, $n = 6$) and VEGF group received intravascular VEGF (0.8 μg/kg) via caudal caval vein (Gr V, $n = 6$). The rest of the groups were subjected to 90 min of ischemia by occlusion of superior mesenteric artery and 4 h of reperfusion. Ischemic reperfusion injury group (Gr I/R, $n = 6$) received no additional medication while the other group received VEGF (0.8 μg/kg) via caudal caval vein at the beginning of reperfusion period (Gr I/R+V, $n = 6$). At the end of reperfusion period, all rats were sacrificed and blood was sampled to evaluate hepatic and renal functions. Also the liver and kidney were harvested in order to evaluate malondialdehyde (MDA), glutathione (GSH), total nitrate/nitrite (NO_x) levels and superoxide dismutase (SOD) and catalase (CAT) activities. The

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Kruskal-Wallis test and Mann-Whitney *U*-test with Bonferroni correction were used for statistical analysis.

The administration of VEGF at the beginning of reperfusion caused decreases in MDA levels and SOD activities and increases in GSH levels and CAT activities for kidney and liver tissues when compared to ischemia reperfusion group. Similarly VEGF at the beginning of reperfusion enhanced renal functions.

The remote organ effects of intestinal IRI can be prevented by VEGF as it presents antioxidant effects in addition to its angiogenesis promoter, and vasodilator effects.

Keywords: ischemic reperfusion injury, VEGF, mesenteric artery occlusion, liver, kidney, antioxidants, oxidative stress.

AIMS AND BACKGROUND

Ischemic reperfusion injury (IRI) is a complex process involving a number of cytokines, chemokines, complements factors, lipid mediators and oxygen radicals and represents a great challenge in clinical practice. At the site of the affected area, the early phase of injury is related to parenchymal damage due to hypoxia, related ATP depletion and intracellular acidosis and the late phase of injury is related to additional damage due to inflammation. The evidence supports both local and systemic effects of IRI as many injurious substances and factors are activated during IRI and released to systemic circulation via venous and/or lymphatic routes[1]. The remote organ injury is inevitable as a component of systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS) and multi-organ dysfunction syndrome (MODS) (Ref. 1).

Mesenteric IRI is a clinical entity associated with a variety of life threatening conditions such as acute mesenteric artery occlusion, intussusception, transplantation, acute blood loss and traumas. The occlusion of superior mesentery artery (SMA) is related to intestinal ischemia and aforementioned changes at the local and systemic level. Moreover, intestinal ischemia causes stagnation in the bowel and damages the intestinal barriers. These changes cause elevated portal endotoxin levels and direct hepatic injury[2,3].

Vascular endothelial growth factor (VEGF) is a mitogenic, angiogenic, vasodilatory protein[4]. As a drug, it has been shown to be effective in treatment of experimental IRI (Refs 5 and 6). In this study, we aimed to evaluate the remote organ effects of VEGF following intestinal IRI.

EXPERIMENTAL

SUBJECTS

The study protocol was approved by Gazi University, Animal Ethics Committee and performed at the Experimental Research Centre of Gazi University, School of Medi-

cine according to the guidelines of the Research Committee of the faculty. The study comprised 24 male adult Wistar-Albino rats with a mean weight of 240 ± 10 g. All animals were kept under controlled temperature (21 ± 2ºC) and humidity (55.5%) with 14 h light and 10 h dark cycle. They were fed with standard rat chaw and free access to water. There were no water and light restrictions throughout the experiment. All animals received humane care in compliance with 'Principles of Laboratory Animal Care' formulated by National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health. All surgical procedures were performed by the same surgeon in the sterile conditions. Every surgical intervention was performed in absolute sterile conditions under 80 mg/kg ketamine hydrochloride (Ketalar, Eczacibasi, Turkey) and 10 mg/kg xylazine hydrochloride (Alfazyne, Ege Vet, Turkey) anesthesia.

The rats were randomly assigned into 4 groups: sham group (Gr S, $n = 6$) underwent a median laparotomy and dissection of superior mesentery artery (SMA) with no further intervention; VEGF group (Gr S, $n = 6$) underwent a median laparotomy and dissection of SMA. After vascular dissection, VEGF (0.8 µg/kg) was administered via caudal caval vein; ischemia/reperfusion group (Gr I/R, $n = 6$) underwent a median laparotomy. After the dissection of SMA, the artery was occluded adjacent to its root by a microclamp for 90 min. The paleness of the jejunoileal segments along with pulseless mesenteric artery confirmed the ischemia; ischemia/reperfusion+VEGF group (Gr I/R+V, $n = 6$) underwent a median laparotomy, dissection, and occlusion of SMA for 90 min. For this treatment group VEGF (0.8 µg/kg) was administered via caudal caval vein at the beginning of reperfusion period.

After 4 h of reperfusion, all rats were sacrificed after removal of kidney and liver and withdrawal of cardiac blood. Blood samples were collected in ethylene diamino tetra acetic acid (EDTA) tubes and centrifuged as soon as possible at 3.000 ×*g* for 10 min at 4ºC. Plasma samples were stored at –80ºC prior to the analyses. Kidney and liver were removed, washed in cold 0.9% NaCl, wiped, weighed and frozen in liquid nitrogen and kept frozen –80ºC until their usage. Plasma lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine levels were calculated with autoanalyser. Renal and hepatic malondialdehyde (MDA), glutathione (GSH), total nitrate/nitrite (NO_x) levels and superoxide dismutase (SOD) and catalase (CAT) activities were calculated with calorimetric tests.

BIOCHEMICAL ANALYSIS

Determination of plasma LDH, AST, ALT, BUN, and creatinine levels. Determination of plasma LDH, AST, ALT, BUN, and creatinine levels was performed using ROCHOP800 autoanalyser.

Determination of tissue MDA levels. MDA (the lipid peroxidation end product) levels were determined using the thiobarbituric acid (TBA) test based on the spectrophotometric measurement of the concentration obtained from the end product of the reaction between lipid peroxides and TBA (Ref. 7). Renal and hepatic tissues were weighed, diluted with RIPA buffer and homogenised at a ratio of 1/5 (w/v). Homogenates were centrifuged at 1.600 × *g* for 10 min at +4ºC. Tissue MDA levels were calculated using Cayman TBARS assay kits at 530 nm and presented as nmol/g tissue.

Determination of tissue protein levels. Tissue protein levels were determined by using Bradford method, a fast and sensitive method for protein binding, using bovine serum albumin (BSA) as the standard[8].

Determination of tissue GSH levels. The Ellman reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) was used to quantify the number of alifatic thiol groups in the sample. At mild alkali pH, tissues reacted with the Ellman reagent and *p*-nitrophenol anion per every thiol group was calculated with spectrophotometry[9]. Renal and hepatic tissues were weighed, diluted with 50 mM metaphosphoric acide (pH 6–7, containing 1 mM EDTA) and homogenised at a ratio of 1/5 (w/v). Homogenates were centrifuged at 10.000 × *g* for 15 min at +4ºC. Tissue GSH levels were calculated using Cayman GSH assay kits at 410 nm and presented as nmol/mg protein.

Determination of tissue NO_x levels. The assay is based to the conversion of nitrate to nitrite by using nicotinamide adenine dinucleotide phosphate (NADPH) with existence of nitrate reductase. Thereafter nitrite reacts with N-(1-naphtyl) ethylene diamine and sulphanomide and the absorbance of end product is calculated at 540 nm (Ref. 10).

Renal and hepatic tissues were weighed, diluted with PBS (pH 7.4) and homogenised at a ratio of 1/5 (w/v). Homogenates were centrifuged at 10.000 × *g* for 20 min at +4ºC. Tissue NO_x levels were calculated using Cayman nitrate/nitrite assay kits at 540 nm and presented as μM.

Determination of tissue SOD activity. The principle of the assay is the degradation of tissue superoxide by SOD enzyme and the production of O_2^- with xanthine oxidase. After the reaction with nitroblue tetrazolium (NBT), the change in colour due to end product was determined spectrophotometrically[11].

Renal and hepatic tissues were weighed, diluted with 20 mM HEPES (pH 7.2, containing 1 mM EDTA, 210 mM mannitol, 70 mM sucrose) and homogenised at a ratio of 1/5 (w/v). Homogenates were centrifuged at 1.500 × *g* for 5 min at +4ºC. Tissue SOD activities were calculated using Cayman SOD assay kits at 440 nm and presented as U/mg protein.

Determination of tissue CAT activity. The calculation is based on the degradation of hydrogen peroxide[12]. Renal and hepatic tissues were weighed, diluted with 50 mM potassium phosphate buffer (pH 7, containing 1 mM EDTA) and homogenised at a ratio of 1/5 (w/v). Homogenates were centrifuged at 10.000 × *g* for 15 min at +4ºC.

Tissue CAT activities were calculated using Cayman CAT assay kits at 540 nm and presented as nmol/min/mg protein.

STATISTICAL ANALYSIS

All statistical analyses were performed by using the statistical package SPSS for Windows, version 15.0 (SPSS, Chicago, Illinois, USA). The descriptive statistics were expressed as the groups mean ± SD. Statistical analyses were carried out using the Kruskal-Wallis test and Mann-Whitney *U*-test with Bonferroni correction. The value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

All animals survived throughout the experiment. The results were represented in Tables 1–3.

Table 1. ALT, AST, LDH, BUN and creatinine levels in the plasma of all the groups (mean ± SD)

Groups	Sham (*n*=6)	VEGF (*n*=6)	I/R (*n*=6)	I/R+VEGF (*n*=6)
ALT (U/l)	66.83±23.91[a, b]	86.83±20.56	119.50±51.97[a]	137.75±53.61[b]
AST (U/l)	423.50±171.77	493.83±141.46	579.5±115.88	438.0±117.95
LDH (IU/l)	1895.33±321.78	1278.66±293.98	1300.16±934.94	1911.16±281.59
BUN (mg/dl)	48.48±6.94[a, b]	43.28±6.17	100.31±21.35[a]	89.0±14.85[b]
Creatinine (mg/dl)	0.20±0.06[a, b]	0.25±0.03	0.40±0.09[a, c]	0.28±0.04[b, c]

[a]ALT, BUN – creatinine (I/R group versus sham group) ($p < 0.05$); [b]ALT, BUN – creatinine (I/R+VEGF group versus. sham group) ($p < 0.05$); [c] creatinine between (I/R+VEGF group versus I/R group) ($p < 0.05$).

Table 2. MDA, GSH, NO_x levels and SOD and CAT activities in the kidney of all the groups (mean ± SD)

Groups	Sham (*n*=6)	VEGF (*n*=6)	I/R (*n*=6)	I/R+VEGF (*n*=6)
MDA (nmol/g)	15.58±2.36[a]	12.49±1.65	22.47±3.97[a, b]	6.78±2.46[b]
GSH (nmol/mg protein)	10.14±3.26	6.94±1.30	5.24±2.89	7.63±1.01
NO_x (μM)	14.78±3.99[a, c]	12.67±3.63[d, e]	33.92±14.61[a, d]	23.61±3.62[c, e]
SOD (U/mg protein)	13.84±5.23[a]	19.07±5.49[d]	7.61±1.29[a, b, d]	20.05±6.86[b]
CAT (nmol/min/mg protein)	27.03±7.67	28.46±8.05	32.75±5.86[b]	20.00±1.36[b]

[a]MDA, NO_x, SOD (I/R group versus sham group) ($p < 0.05$); [b]MDA, SOD, CAT (I/R+VEGF group versus I/R group) ($p < 0.05$); [c] NO_x (I/R+VEGF group versus sham group) ($p < 0.05$); [d] NO_x, SOD (VEGF group versus I/R group) ($p < 0.05$); [e] NO_x (I/R+VEGF group versus VEGF group) ($p < 0.05$).

Table 3. MDA, GSH, NO_x levels and SOD and CAT activities in the liver of all the groups (mean±SD)

Groups	Sham (*n*=6)	VEGF (*n*=6)	I/R (*n*=6)	I/R+VEGF (*n*=6)
MDA (nmol/g)	9.46±1.18[a]	12.13±2.11[b]	23.11±5.91[a, b, c]	7.28±2.06[c]
GSH (nmol/mg protein)	14.89±2.37	14.51±6.04	10.74±4.25	15.79±3.41
NO_x (μM)	23.10±6.28	24.00±5.41	25.73±5.81	24.06±4.18
SOD (U/mg protein)	21.07±5.35[a]	17.45±5.11	12.23±2.16[a, c]	17.06±2.47[c]
CAT (nmol/min/mg protein)	30.78±6.47	31.10±6.46	24.27±5.78	32.4±11.51

[a]MDA, SOD (I/R group versus sham group) ($p < 0.05$); [b]MDA (VEGF group versus I/R group) ($p < 0.05$); [c]MDA, SOD (I/R+VEGF group versus I/R group) ($p < 0.05$).

Hepatic tests. ALT level was found to be significantly higher in I/R, and I/R+V groups when compared to the sham and drug groups ($p < 0.05$). No significant decrease for ALT was observed in the treatment group. Also, no statistical significant differences were found among the groups in terms of AST and LDH levels.

Renal tests. BUN and creatinine levels were found to be significantly elevated in I/R, and I/R+V groups when compared to the sham and drug groups ($p < 0.05$). Only the creatinine level was found to be significantly decreased in I/R+V group when compared to the I/R group ($p < 0.05$).

MDA levels. The MDA levels in renal tissues were found to be elevated in I/R group when compared to the sham group ($p < 0.05$). Even though all groups treated with drugs (V, and I/R+V) demonstrated lower MDA levels in renal tissue, the decrease in the Gr I/R+V was found to be statistically significant when compared to the Gr I/R ($p < 0.05$). The MDA levels in hepatic tissues were found to be elevated in I/R group when compared to the other groups ($p < 0.05$). Also the MDA level in liver tissue was found to be significantly elevated in Gr V ($p < 0.05$). Hepatic MDA level was found to be significantly decreased for treatment groups when compared to the Gr I/R ($p < 0.05$).

GSH levels. The GSH levels in renal tissues were found to be decreased in I/R group when compared to the other groups ($p > 0.05$). Even the treatment groups demonstrated higher GSH levels, those differences were statistically insignificant. The GSH levels in liver tissue were also found to be decreased in Gr I/R ($p > 0.05$). Gr I/R+V demonstrated higher GSH levels. But those differences were statistically insignificant.

NO_x levels. The NO_x levels in kidney tissue were found to be elevated in all I/R groups (I/R, and I/R+V) when compared to the other groups ($p < 0.05$) but no change was found among I/R groups. No statistically significant change was determined for hepatic NO_x levels among all groups.

SOD activity. The SOD activities in renal tissues were found to be decreased in I/R group when compared to the other groups ($p < 0.05$). Renal SOD activities were found to be elevated for the Gr I/R+V ($p < 0.05$). The SOD activities in liver tissue were

also found to be decreased in the Gr I/R when compared to the sham group ($p < 0.05$). The increase in hepatic SOD activities of the Gr I/R+V was statistically significant when compared to the Gr I/R ($p < 0.05$).

CAT activity. Despite no difference observed between S, and I/R groups in terms of CAT activities in kidney tissue, the renal CAT activities were found to be decreased in the Gr I/R+V when compared to the Gr I/R ($p < 0.05$). Even when a decrease of CAT activities in liver tissue was observed for Gr I/R, no statistically significant difference was determined among the groups. VEGF is an important signal protein that stimulates angiogenesis following vascular injuries. The activity of blood vessels growth from pre-existing vasculature is of importance especially when the blood circulation is not adequate. The hypoxia is the major stimulant to produce VEGF and its receptors and contributes to angiogenesis[13]. As a drug, it has been shown to be effective in treatment of experimental IRI (Refs 5 and 6). In a study by Luo et al.[14], VEGF was demonstrated to modulate various aspects of endothelial function and repair besides its angiogenesis promoter effect. Moreover, Oyar et al.[6] demonstrated its antioxidant effect in an experimental model of ischemic spinal cord injury. In this study, we aimed to evaluate the remote organ effects of VEGF following intestinal IRI and found that VEGF alleviates the remote organ injury following intestinal IRI with its antioxidant effects in addition to its angiogenesis promoter, and vasodilator effects. In this study, experimental intestinal IRI was achieved as described by Dwivedi et al.[15], Sizlan et al.[16] evaluated the remote organ effects of proanthocyanidin in an intestinal IRI model of 60 min ischemia and 6 h reperfusion. They stated that 60 min of ischemia are not enough to produce adverse effects on kidney and liver as these organs are more resistant than any other tissue. Moreover, the liver has a strong antioxidant capacity of its own[17]. Varga et al.[18] also studied the remote organ effects of IRI on acute and subacute phases and revealed that the peak alteration at tissue level was achieved at the 4th h of reperfusion[18]. Due to these reasons, despite the controversies in timetables presented in the literature for IRI, we chose 90 min ischemia and 4 h reperfusion period to see the remote organ effects.

VEGF is a member of platelet derived growth factor super family[19]. It is an important signal protein during vasculogenesis and angiogenesis. This protein is also referred as 'vascular permeability factor' as it stimulates the production of nitric oxide (NO), causes vasodilation and increases microvascular permeability[4]. Intravenous VEGF dose of 0.8 μg/kg was proven to be the minimum effective concentration in an earlier study[6].

We aimed to evaluate the effects of intestinal IRI on glomerular hemodynamics, and hepatic functions. Both functions were found to be deteriorated related to IRI and drug administration only alleviated creatinine levels. VEGF was not found to reverse the deterioriation as the hepatic damage is still ongoing due to both portal endotoxemia and inflammatory process directed towards hepatic route.

Free oxygen radicals resulted by IRI reacts with polyunsaturated fatty acids of cell membrane phospholipids and causes lipid peroxidation. MDA is a marker of oxidative stress and can express the degree of membrane damage[20,21]. Both hepatic and renal MDA levels that were found to be significantly elevated following IRI and VEGF treatment has decreased those levels. These findings suggested that VEGF administration reverses the lipid peroxidation in remote organs following intestinal IRI.

Despite its key roles during IRI, the effects of NO during this process are controversial. NO reacts with superoxide anion to form a potent oxidant substance that has an active role in hypoxia re-oxygenisation process. Inducible NO synthase (iNOS) however worsens the IRI and iNOS inhibitors reverse this damage, endothelial NO synthase enhances the IRI via different cascades including vascular regulation[22,23]. In this study, we found that renal, not hepatic NO_x levels, were elevated with intestinal IRI and VEGF treatment has not affected the damage. NO is an important IRI marker especially for liver. The early phase of IRI demonstrates the cytotoxic effects of NO whereas late phase of IRI is related with endogenous production of NO which has cytoprotective effects[24]. But the duration of reperfusion is found to be indirectly proportional to NO production[25]. Our results indicated no significant NO_x change at liver, probably as the antioxidant capacity of liver is beyond these limits.

The endogenous enzymes, SOD and CAT act together, degrade superoxide radical and protect the cell from oxidative stress. The fluctuations of SOD and CAT levels during IRI in diverse studies are attributed to either depletion or overproduction of enzymes to overcome the increased level of superoxide during increased oxidative stress[26–28]. The changes demonstrated with the endogeneous enzymes are accepted as consumption of SOD due to injury and VEGF treatment resulted in elevation of SOD suggesting antioxidant effects of VEGF. GSH is another antioxidant agent that depletes during IRI. Nijmeh et al.[29] studied on ischemia induced angiogenesis models and revealed decreases in GSH levels following ischemia. We determined decreases for liver and kidney GSH levels following IRI with no permenant change with treatment.

Ischemia-reperfusion injuries are considered to increase oxidative stress in whole body. It was demonstrated that plasma and spinal cord levels of NO, MDA, and advanced oxidation protein products (AOPP) levels were significantly increased after spinal cord injury in New Zealand rabbits. The plasma and spinal cord NO, MDA, and AOPP levels were decreased by the administration of lipoic acid (LA) after spinal cord injury[30].

Yildirim et al.[31] demonstrated that the MDA and tumor necrosis factor-α (TNF-α) levels were significantly higher in the ischemia group when compared to the control group. The vitreous MDA and TNF-α levels were significantly lower in the LA group when compared to the ischemia group. There was no significant difference between the vitreous total antioxidant status (TAS) levels of the groups.

CONCLUSIONS

In this study, we demonstrated liver and kidney damage following intestinal IRI and VEGF might be beneficial for treatment as it reversed these damages. As VEGF is known to stimulate NO production, cause vasodilation and increase microvascular permeability, it might be a good option for such an injury in clinical settings. Moreover, we represented antioxidant effects of VEGF which in return supports the drug for consideration in clinical practice.

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