Role of Estrogen in Acute Ischemic Reperfusion Renal Injury in Adult Female Rats and its Possible Underlying Mechanisms of Action

MAHA M. GAMAL EL-DIN, M.D.*; HEBA M. SHAWKY, M.D.*; MONA M. MOHAMED, M.D.*; MOHAMED A. ESHRA, M.B.B.Ch* and LEILA A. RASHED, M.D.**

The Departments of Physiology* and Medical Biochemistry**, Faculty of Medicine, Cairo University

Abstract

Renal ischemic reperfusion Injury (I/R) is an important clinical problem that shows sex differences thus there was a specific role of sex steroids in renal injury. This study aimed to investigate the protective effect of estrogen as an anti-inflammatory and anti-oxidant agent in renal I/R injury. Five groups were included (n=10/group): Sham-operated, I/R of the kidney, I/R after bilateral ovariectomy, I/R after bilateral ovariectomy and estrogen supplementation and I/R after bilateral ovariectomy with estrogen supplementation and L-NAME. When compared with the sham-operated group, I/R resulted in significant increase in the serum creatinine (0.46 ± 0.17 mg/dl versus 0.15 ± 0.06 mg/dl), rat tail systolic B.P. (116.9 ± 5.09 mmHg versus 110.2 ± 2.44 mmHg), p-38 MAPK (0.82 ± 0.12 versus 0.27 ± 0.09) and peroxynitrite level (186.08 ± 21.9 nmol/mg ptn versus 101.25 ± 11.09 nmol/mg ptn) and significant decrease in e-NOS (0.6 ± 0.38 versus 1.98 ± 0.58), bilateral ovariectomy with estrogen supplementation resulted in significant decrease in serum creatinine (0.5 ± 0.17 mg/dl versus 0.82 ± 0.13 mg/dl), rat tail systolic B.P. (116 ± 3.06 mmHg versus 121.4 ± 6.35 mmHg), p-38 MAPK (0.6 ± 0.16 versus 1.30 ± 0.34) and peroxynitrite (162.89 ± 21.04 versus 204.24 ± 12.41) and significant increase in e-NOS (1.347 ± 0.42 versus 0.22 ± 0.08) compared to I/R after ovariectomy. In Conclusion: Estrogen provided a protective role against renal I/R injury and subsequent dysfunction as it produced an anti-oxidant impact mediated by increased expression of e-NOS and decreased peroxynitrite in renal tissue and anti-inflammatory effects manifested by decreased expression of p38 MAPK in renal tissue. L-NAME blocking the anti-oxidant effect of estrogen suggests that it may be mediated through NO action.

Key Words: I/R – Estrogen – L-NAME – P-38 MAPK – Peroxynitrite – e-NOS.

Introduction

There is growing evidence that significant sex differences exist in the response of the kidney to injury and there is current clinical evidence of increased male susceptibility to acute and chronic renal injury.

Endothelial dysfunction which is an important component of initiating and continuing renal tubular epithelial injury and contributes to the pathogenesis of ischemic acute renal failure (ARF) [19]. Endothelial injury may aggravate the inflammatory response through loss of normal nitric oxide (NO) production due to inhibition of endothelial nitric oxide synthase (eNOS). The produced NO reduces leukocyte-induced injury by blocking leukocyte sequestration and activation.

However, I/R also increases inducible nitric oxide synthase (iNOS), which potentiates injury [4,14] as the nitric oxide produced reacts with oxygen radicals to form peroxynitrite, which could induce protein nitration. One of the consequences of protein nitration could be the nitration of actin tyrosine residues in the cytoskeleton, thus possibly inducing cytoskeletal alterations [34]. Also, the high output production by iNOS might suppress eNOS [30]. This imbalance between the two NO synthases may be an important component of renal I/R injury.

The incidence of chronic renal disease in women increases with aging, especially after menopause, suggesting that loss of sex hormones may contribute to the development and progression of renal diseases. However, the mechanisms by which sex hormones, particularly estrogens, contribute to the disease process are unclear [16].

Park et al. [22] showed that after renal I/R iNOS activity increased in both males and females while eNOS activity decreased in males but increased in females. Therefore, this difference in eNOS activity may be an important component of the gender differences observed in the response of men and women to renal I/R injury.
difference noted, since it was showed that estrogen in physiologic concentrations activates eNOS in a biphasic manner [31]. The initial increase is mediated by ERK [21], while the second increase occurs through phosphatidylinositol 3-kinase (PI3K) [31], both of which are activated through the rapid, non-genomic actions of estrogen. ERK is probably activated through the Src/Shc/ERK pathway while estrogen receptor alpha interacts with a subunit of PI3K and stimulates it leading to activation of both eNOS and Akt. Thus, estrogen may mediate the gender differences in renal I/R through differences in eNOS expression and NO production. Also 17beta-estradiol (E2-beta) prevents renal injury and suppresses renal endothelin-1 overproduction in ischemic acute renal failure. NO may be closely related to suppressive effect of E2-beta on renal endothelin-1 overproduction in rats with acute renal failure rats [30].

The MAPK family is divided into 3 subfamilies: extracellular regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. These MAPKs are activated by I/R injury not only in the kidney but also in other organs. Park et al. [22] found JNK is activated with ischemia but more so in males than females. Castration reduced JNK activation in males but did not alter it in females furthermore, estrogen administration to males was observed to lower JNK activation. They also found that I/R increases ERK phosphorylation in females more than in males. In ovariectomy ERK activation was reduced, while castration did not alter it, suggesting that estrogen may be more important in mediating sex differences in ERK activation.

Wang et al. [36] also reported that females had lower p38 MAPK activation after myocardial I/R and which was associated with lower proinflammatory cytokine production and better recovery of myocardial function.

In view of all these studies, greater understanding of the specific role of sex steroids in renal injury may provide new therapeutic strategies to protect against inflammatory injury and renal damage in the future.

The aim of this work is to investigate the protective effect of estrogen as an anti-inflammatory and anti-oxidant agent in I/R renal injury and its possible mechanisms of action, by studying e-NOS gene expression, p38 MAPK (inflammatory mediator and pro-apoptotic agent) gene expression and peroxynitrite (oxidative stress marker) levels in the renal tissue in I/R injury and their modulation by estrogen.

Material and Methods

Experimental animals and protocol:
A total of fifty of 10-12 weeks old adult female Wistar rats weighing 150-200gm, were housed in wire mesh cages under constant room conditions (temperature 22-24°C, alternating 12:12h light-dark cycle) and had free access to food and water. Veterinary care was provided by the laboratory Animal House Unit of Faculty of Medicine, Cairo University. Rats were allowed to acclimatize to their environment for five days before the start of the experiments. All experimental procedures were carried out in compliance with the guide for care and use of laboratory animals published by the US National Institutes of Health (NIH publication 85-23 revised 1985) and in compliance with the Local Animal Ethics Committee of Kasr Al Aini, Faculty of Medicine, Cairo University. The work was done during 2010.

The animals were then divided into five groups (n=10/group):

Group I : Control (sham-operated rats).

Group II : I/R of the kidney (ischemia for 50 minutes then reperfusion for 2 hours) [20].

Group III: I/R of the kidney was induced after one month of bilateral ovariectomy (OX) [13].

Group IV: Ovariectomized rats were supplied with estrogen supplementation (E2 in a dose of 25µg/kg/day SC) for 3 weeks which started 7 days after bilateral OX [26], after which I/R of the kidney was performed.

Group V : Ovariectomized rats supplied with estrogen (E2 25µg/kg/day SC) and co-supplemented with L-NAME (0.3mg/kg/day IV) for 3 weeks after 7 days of bilateral OX [30] then I/R of the kidney was done.

At the end of the experimental protocol i.e (2 hours after reperfusion) blood samples were taken for assessment of serum creatinine and blood urea. In addition rat tail systolic blood pressure was measured. Endothelial NOS gene expression, P38 MAPK gene expression and Peroxynitrite levels were estimated in renal tissue.

Chemicals:
- Estrogen was obtained from Sigma (St. Louis, MO, USA) in the form of ampoules, every 1ml of the ampoule contains 5mg estradiol benzoate.
Each of which was dissolved in 200ml oily solution. Control rats only received an oily solution as a vehicle (1ml/kg/day SC).

- L-NAME was obtained from Sigma (St. Louis, MO, USA) in the form of powder and was dissolved in isotonic saline solution (3mg powder/10ml saline). The vehicle-treated rats representing the control group were injected the same amount of isotonic saline only (1ml saline/kg/day IV).

- Thiopental sodium was obtained from Eipico Co., Egypt in the form of vials, each containing 500mg of the ingredient.

Surgical procedures:

Ovariectomy:

The rats were anesthetized by intraperitoneal injection with thiopental sodium (30mg/kg), then a longitudinal incision was made in the midline of the animal back just caudal to 13th rib; to expose each ovary. The ovaries were removed and the wound was then closed using a 4-0 sterile suture. The rats were returned to their cages and left for about 7 days for recovery (6).

Renal I/R injury animal model:

Rats were anesthetized by intraperitoneal injection with thiopental sodium (30mg/kg). A midline abdominal incision was made and both kidneys were exposed. Renal ischemia was induced by nontraumatic vascular clamps over the pedicles (arteries and veins) of the two kidneys for 50min. Following the occlusion, the presence of ischemia was visually confirmed by observing the blanching of the kidneys. During the period of ischemia, the edges of the abdominal incision were approximated to each other and covered by a cotton pad soaked with warm isotonic saline (37°C) to prevent undue loss of body fluids. After removal of the clamps, the kidneys were observed for an additional 1 minute to observe the color change indicative of blood reflow, after which the incision was closed in two layers with 2-0 sutures [17].

Sham operation:

Sham-operated animals received equivalent anesthesia and underwent laparotomy but without clamping.

Mortality rate:

Three rats died during the surgical procedures.

Immediately before sacrifice, rat tail blood pressure was measured and blood samples were withdrawn through retro-orbital route using heparinized capillary tubes then placed in 10ml eppendorf tubes. The blood samples were centrifuged for further determination of serum creatinine and urea. After the animals were sacrificed by cervical dislocation tissue samples from kidney were dissected and kept frozen at -80°C in liquid nitrogen.

Blood pressure measurement:

Systolic blood pressure in rats was measured by the Harvard rat tail blood pressure monitor system. A hand inflation bulb is used to inflate the cuff that fits over the tail to occlude the blood flow, while the optical pick up detects blood flow. A pressure transducer in the control unit converts the pressure to an analogue signal for recording, i.e., changes in the blood flow are detected by the optical pick up unit and these changes are transmitted to a pressure transducer located in the control unit of the apparatus. The control unit then converts the pressure signals to electronic analogue signals which are displayed on a PC computer system. At least three consecutive recordings were taken. If these three recordings were similar, then their value was taken as systolic blood pressure measurement.

Biochemical analysis:

- Serum creatinine was estimated using QuantiChromTM creatinine Assay Kit.
- Detection of eNOS, p38 MAP kinase and beta actin gene expressions by Polymerase Chain Reaction (PCR).

Total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform method [5]. RNA content and purity were measured using ultraviolet spectrophotometer (A260/A280 ratio of 1.8-2.0). For amplification of the target genes, reverse transcription-PCRs were run in two separate steps. In brief, equal amounts of total RNA (6mcg) were heat denatured and reverse transcribed by incubating at 42 1C for 90min with 12.5U (avian myeloblastosis virus) reverse transcriptase (Promega, Madison, WI, USA), 200nM deoxyribonucleotide triphosphate mixture and 1nM oligo-dT primer in a final volume of 30ml of 1 avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA), 200nM deoxyribonucleotide triphosphate mixture and 1nM oligo-dT primer in a final volume of 30ml of 1 avian myeloblastosis virus buffer. The reactions were terminated by heating at 97 1C for 5min and cooled on ice. Complementary DNA samples were amplified in 50ml of 1_ PCR buffer in the presence of 2.5U Taq DNA polymerase (Promega), 200nM deoxyribonucleotide triphosphate mixture and appropriate primer pairs (1nM of each primer). PCR consisted in a first denaturing cycle at 97 1C for 5min, followed by a variable number of cycles of amplification defined by denaturation at 96 1C for 1.5min, annealing for 1.5min and extension at 72 1 C for 3min. A final extension cycle of 72 1 C for 15min was included. PCR products were electro-
phoresed on 2% agarose stained with ethidium bromide and visualized by ultraviolet transilluminator. Semiquantification was performed using gel documentation system (BioDO, Analyser, Biometra, Göttingen, Germany). According to the amplification procedure, relative expression PCR detection of β-actin.

Presence of RNA in all samples was assessed by analysis of the ‘house-keeping’ gene β-actin. Complementary DNA was generated from 1mg total RNA extracted with avian myeloblastosis virus reverse transcriptase for 60min at 37 1C. For PCR, 4ml complementary DNA was incubated with 30.5ml water, 4ml 25mM MgCl2, 1ml deoxyribonucleotide triphosphates (10mM), 5ml 10_ PCR buffer, 0.5ml (2.5 U) Taq polymerase and 2.5ml of each primer containing 10pM. β-actin primers (forward 50-TGGTTGCTCTGTATGCCT CT-3'; reverse 5-TAATGTCACGCAGATTTCC-3') was designed from GenBank (accession no. J00691). The reaction mixture was subjected to 40 cycles of PCR amplification, denaturation at 95 1C for 1 min, annealing at 57 1C for 1 min and extension at 72 1C for 2min. Primer sequences were as follows: eNOS forward primer 5’-ATA GAA GAA GGG AAA TAG CAA ATG G-3’, reverse primer 5’-TTA ACG ATG GAG TCC AAG CGC A-3’, MAP38 forward primer 5’-AGA GCG CCA GCC CTC TGA CGT CCA T-3’ and reverse primer 5’-TCC GTT TCC TGC AGC AGT CTC CGC A-3’.

- Peroxynitrite detection was done using hydroxyl/peroxynitite detection kit tm supplied by cell technology incorporation California (USA) according to manufacturers instruction.

Statistics:

The results were analysed using SPSS computer software package version 15. Data were presented as mean ± SD and evaluated by one-way ANOVA followed by post hoc, Kruskal-Wallis & Mann-Whitney tests. Differences of p<0.05 were considered significant [1].

Results

Assessment of kidney function:

As Revealed in Fig. (1):

Serum creatinine was significantly elevated in I/R group (group II) and in I/R after OX (group III) compared to control group (group I) (p<0.05). Serum creatinine in I/R after estrogen supplementation (group IV) showed significant decrease compared to OX group (group III) (p<0.05). Whereas it showed no significant difference compared to I/R group (group II) (p>0.05). Serum creatinine in I/R after estrogen supplementation and L-NAME treatment (group V) showed significant increase compared to estrogen-supplemented group (group IV) and to I/R group (group II) (p<0.05), but no significant change was recorded compared to OX group (group III) (p>0.05).

Rat tail systolic blood pressure in the studied group:

As Observed in Fig. (2):

Rat tail systolic blood pressure was significantly elevated in I/R group (group II) and I/R after OX (group III) compared to control group (group I) (p<0.05), whereas, the mean value in I/R after OX (group III) showed no significant increase compared to I/R group (group II) (p>0.05). Rat tail systolic blood pressure was significantly decreased in I/R after estrogen supplementation (group IV) compared to OX group (group III) (p<0.05), however, no statistically significant difference was shown compared to I/R group (group II) (p>0.05). In I/R after estrogen supplementation and L-NAME treatment (group V), it showed significant increase compared to estrogen-supplemented group (group IV) and to OX group (group III) (p<0.05) and when compared to I/R group (group II), it showed significant increase (p<0.05).

The level of e-NOS gene expression in the studied groups:

As Revealed in Fig. (3):

As depicted, the level of e-NOS gene expression showed a significant decrease in I/R group (group II) and in I/R after OX (group III) compared to control group (group I) (p<0.05). Furthermore, in I/R after OX (group III) it also showed significant decrease compared to I/R group (group II) (p<0.05). In I/R after estrogen supplementation (group IV) the levels of e-NOS expression showed significant increase compared to OX group (group III) and compared to I/R group (group II) (p<0.05). These gene expressed levels of e-NOS in I/R after estrogen supplementation and L-NAME treatment (group V), however showed significant decrease compared to estrogen-supplemented group (group IV) (p<0.05), while it showed no significant change compared to OX group (group III) (p>0.05). In I/R in response to estrogen supplementation and L-NAME treatment (group V) the e-NOS levels expressed showed insignificant change compared to I/R group (group II) (p>0.05).

The levels of P38 MAPK gene expression and peroxynitrite in the studied groups:

As Revealed in Figs. (4,5):

As shown in the figures, the levels of P38
MAPK gene expression and peroxynitrite displayed a significant increase in I/R group (group II) compared to control group (group I) \((p<0.05)\). Furthermore, I/R after OX (group III) showed significant increase in P38 MAPK gene expression and in peroxynitrite compared to control group (group I) \((p<0.05)\).

The level of P38 MAPK gene expression in I/R after OX (group III) showed significant increase compared to I/R group (group II) \((p<0.05)\) but the value of peroxynitrite in this group showed no significant change compared to I/R group (group II) \((p>0.05)\). On the other hand, P38 MAPK gene expression and peroxynitrite in I/R after estrogen supplementation (group IV) showed significant decrease compared to OX group (group III) as well as to I/R group (group II) \((p<0.05)\), whereas peroxynitrite showed no significant change in I/R after estrogen supplementation (group IV) compared to I/R group (group II) \((p>0.05)\).

The level of P38 MAPK gene expression in I/R after estrogen supplementation and L-NAME treatment (group V) showed no significant change compared to estrogen-supplemented group (group IV) \((p>0.05)\). However, P38 MAPK gene expression in I/R after estrogen supplementation and L-NAME treatment (group V) showed significant decrease compared to OX group (group III) \((p<0.05)\). In addition, peroxynitrite in I/R after estrogen supplementation and L-NAME treatment (group V) showed significant increase compared to estrogen-supplemented group (group IV), but showed significant decrease compared to OX group (group III) \((p<0.05)\). The levels of P38 MAPK gene expression and peroxynitrite in I/R after estrogen supplementation and L-NAME treatment (group V) showed no significant change compared to I/R group (group II) \((p>0.05)\).

**Correlation between means of the studied groups:**

Strong negative correlation between Serum creatinine and the level of e-NOS gene expression \((R = -0.898 \text{ and } P < 0.05)\), between the level of P38 MAPK gene expression and the level of e-NOS gene expression \((R = -0.893 \text{ and } P < 0.05)\) and between the level of peroxynitrite and the level of e-NOS gene expression \((R = -0.967 \text{ and } P < 0.05)\) but showed positive correlation between the level of P38 MAPK gene expression and peroxynitrite \((R = 0.882 \text{ and } P < 0.05)\) and no correlation was found between Rat Tail Systolic Blood Pressure and the level of e-NOS gene expression, between Rat Tail Systolic Blood Pressure and the level of P38 MAPK gene expression and between Serum creatinine and the level of P38 MAPK gene expression.
Fig. (3): e-NOS gene expression in all the studied groups: The level of e-NOS gene expression was significantly decreased in all groups compared to control group (group I) \((p<0.05)\), in I/R after estrogen supplementation (group IV) it showed significant increase compared to OX group (group III) and compared to I/R group (group II) \((p<0.05)\). The level of e-NOS gene expression in I/R after estrogen supplementation and L-NAME treatment (group V) was significantly decreased compared to estrogen-supplemented group (group IV) \((p<0.05)\).

Asterisk: Significant compared with control group; number sign: Significant compared with I/R of the kidney; @: Significant compared with I/R of the kidney after ovariectomy; dollar sign: Significant compared with ovariectomized rats treated with estrogen supplementation.

Fig. (4): P38 MAPK gene expression in all the studied groups: The levels of P38 MAPK gene expression showed significant increase in all groups compared to control group (group I) \((p<0.05)\), in I/R after estrogen supplementation (group IV), it showed significant decrease compared to OX group (group III) and compared to I/R group (group II) \((p<0.05)\). The level of P38 MAPK gene expression in I/R after estrogen supplementation and L-NAME treatment (group V) showed significant decrease compared to OX group (group III) \((p<0.05)\). Asterisk: Significant compared with control group; number sign: Significant compared with I/R of the kidney; @: Significant compared with I/R of the kidney after ovariectomy; dollar sign: Significant compared with ovariectomized rats treated with estrogen supplementation.

Fig. (5): Peroxynitrite (nmol/mg ptn) in all the studied groups: Peroxynitrite showed significant increase in all groups compared to control group (group I) \((p<0.05)\), in I/R after estrogen supplementation (group IV), it showed significant decrease compared to OX group (group III) \((p<0.05)\), peroxynitrite in I/R after estrogen supplementation and L-NAME treatment (group V) showed significant increase compared to estrogen-supplemented group (group IV) and significant decrease compared to OX group (group III) \((p<0.05)\). Asterisk: Significant compared with control group; number sign: Significant compared with I/R of the kidney; @: Significant compared with I/R of the kidney after ovariectomy; dollar sign: Significant compared with ovariectomized rats treated with estrogen supplementation.
Fig. (6): An agarose gel electrophoresis show PCR products of eNOS (A), P38MAPkinase (B) & beta actin (C) in different studied group, Lane M: DNA marker, Lane 1: PCR products in Control group, Lane 2: PCR products in I/R group, Lane 3: PCR products in OX + I/R group, Lane 4: PCR products in OX + Estrogen + I/R group & Lane 5: PCR products in OX + Estrogen + L-NAME + I/R group.

Discussion

Acute renal failure (ARF) caused by renal I/R is an important clinical problem. Even though great progress has been made in patient care, there is still high morbidity and mortality associated with ARF. Thus, renal I/R injury was considered an important determinant of allograft survival after transplantation [23].

Renal dysfunction was observed to be produced by I/R injury via increase in inflammation and oxidative stress. In the present study, renal I/R resulted in deterioration of renal function, elevation of systolic blood pressure, elevation of p38 MAPK gene expression and peroxynitrite level in renal tissues. Ovariectomy aggravated the I/R injury but estrogen supplementation provided a protective role against I/R as it produced anti-oxidant and anti-inflammatory effects. L-NAME was shown to be capable of blocking this protective effect of estrogen suggesting that this protective effect may be mediated through NO via an increase in e-NOS expression.

Renal I/R injury occurs through a complex interaction between renal hemodynamics and inflammatory mediators. The endothelial and tubular injury and the endothelial injury may aggravate the inflammatory response through loss of normal nitric oxide production due to inhibition of endothelial nitric oxide synthase (eNOS) [4].

The results of the current work demonstrated that the I/R group (group II) exposed to 50min of renal ischemia and 2 hours of reperfusion showed a significant increase in the serum creatinine, rat tail systolic B.P., p-38 MAPK gene expression and peroxynitrite level whereas a significant decrease in renal e-NOS gene expression was observed compared to the control group (group I) \( (p<0.05) \).

The result of the present study was in agreement with the work of Kadkhodaee et al. [10] who reported that both 45 and 60min ischemia followed by 1 hour reperfusion resulted in significant increases in plasma creatinine and blood urea nitrogen (BUN) compared to the sham-operated group. The work of Müller et al. [20] reported that mean arterial pressure was significantly higher in female rats following renal ischemia for 50 minutes and reperfusion for 60 minutes than pre-ischemic pressure. They explained these results as a result of several vasoactive agents such as endothelin being released.
It is worthy to mention that ovariectomy in our study aggravates renal injury, thus I/R after OX group (group III), showed significant increase in the serum creatinine and p-38 MAPK gene expression compared to I/R group (group II) \( (p<0.05) \). These results were consistent with the findings of Hutchens et al. [9] who reported that loss of ovarian steroids resulted in enhanced renal injury (increased BUN and serum creatinine) 24 hours after global model of renal ischemia. As regards the mechanism of increased p-38 MAPK gene expression in renal tissues in the I/R, some studies postulate that it may be the balance between the two (ERK versus JNK/p38) that determines the fate of the cell [37]. Renal I/R causes necrosis predominantly of the proximal tubules (PT), especially the S3 portion [33], while the thick ascending limb (TAL), distal convoluted tubules and collecting ducts are relatively spared. There is increased ERK phosphorylation in TAL but not in PT cells while JNK is activated in both these cells and hence it has been speculated that this difference in the response may be the cause for the relative difference in the survival of these cells. Other studies have also shown that renal I/R induces JNK [24] whereas inhibition of the latter decreases the development of ARF.

To our knowledge no other study investigated p38 MAPK gene expression in renal tissue after OX. However, El Habashi et al. [6] reported that the levels of p38 and TNF-α levels in the hearts of female dogs decreased compared to ovariectomized females subjected to the same I/R injury (25 minutes occlusion of the left anterior descending coronary artery and subsequent reperfusion).

Our study is one of few studies that examined the effect of ovariectomy in I/R on renal e-NOS gene expression in renal tissues in which, it was significantly decreased compared to I/R group \( (p<0.05) \). Kim et al. [11] showed an apparent down regulation of e-NOS expression 4 weeks after bilateral OX and the restoration of expression in the rat urinary bladder by 17β-estradiol treatment suggesting that the cause of down regulation of e-NOS expression after OX is estrogen deprivation.

The vascular endothelial damage occurring after I/R, could cause an impaired vasodilator ability of the renal vasculature due to a decreased endothelial NO generation and may contribute to the reduction in glomerular filtration in recipients of cadaveric renal allografts [13].

The present results also are in accordance with those performed by Prathapasinghe et al. [25] who found that the level of two biochemical markers for oxidative stress (peroxynitrite, lipid peroxide) and a renal functional marker (plasma creatinine level) were increased in rats subjected to renal ischemia for 1 hour and reperfusion for 1 hour. They found that administration of anti–homocysteine antibodies to kidneys effectively abolished I/R-induced peroxynitrite formation and lipid peroxidation during the first hour of reperfusion. They reported that elevation of renal superoxide was observed only during the reperfusion period and that superoxide reacts with NO to form peroxynitrite, which when increased is believed to be responsible for I/R-induced oxidative stress in the kidney.

As regard rat tail systolic B.P., it was not significantly different in the I/R after OX group compared to the I/R group \( (p>0.05) \). Yet the present findings are controversial with study of Mercier et al. [18] who reported that in 3 and 6 week ovariectomized female Sprague-Dawley rats, uterus atrophy was associated with a significant increase in mean arterial pressure. They also mentioned that endothelin-1 acting via the stimulation of the ETA receptor represents an integral mechanism implicated in the increase of mean arterial pressure following ovariectomy. This conflict may be due to different animal species used.

In our study the main objective is to assess the protective role of estrogen as an anti-inflammatory and anti-oxidant agent in renal I/R. Serum creatinine and rat tail systolic B.P in I/R after estrogen supplementation (group IV) decreased significantly whereas e-NOS gene expression levels increased significantly compared to OX group (group III) suggesting that estrogen supplementation may provide protection against I/R renal injury \( (p<0.05) \).

NO production is better preserved in females than in males, partly as a result of the actions of estrogens. Estrogen may induce renoprotective effects due to its activation of the PI3K/Akt pathway followed by increased e-NOS phosphorylation in the post-ischemic kidney [27].

As a result of I/R injury, P38 MAPK gene expression in renal tissues in response to estrogen supplementation, it decreased significantly compared to OX vehicle-treated group (group III) and to I/R group (group II) suggesting that estrogen in the present study acted as an anti-inflammatory agent by inhibiting the expression of p38 MAPK.

No other study so far, investigated the effect of estrogen on p38 MAPK gene expression in renal tissue. However, El Habashi et al. [6] reported that
endogenous estrogen may be partly responsible for cardioprotective effect in a myocardial I/R dog model by lowering p38 MAPK activation and subsequently by decreasing TNF-α production and this beneficial effect is lost in ovariectomized females. They also reported that estrogen may reduce p38 MAPK activity through decreased formation of free radicals or through increased formation of NO estrogen mediated.

In I/R, Peroxynitrite level in renal tissues after estrogen supplementation (group IV) decreased significantly compared to OX group (group III) \((p<0.05)\), thus demonstrating an anti-oxidant role of estrogen in I/R injury. To date, few in vivo studies have directly probed the E2’s role in oxidative stress resistance. The only mouse study in this issue was done by Baba et al. \[2\] in wild-type and insulin receptor hemizygous null (IR\(^{-/-}\)) mice. In the course of studying the gender dimorphic effect of the mutant IR allele’s protection against oxidative stress, they found that ovariectomized female mice of both genotypes were more susceptible to hyperoxia and that their resistance to it could be rescued by E2 supplementation. This was accompanied by changes in the expression and activity of manganese superoxide dismutase. Also Kozlov et al. \[12\] found that E2 has an anti-oxidant effect on rat liver following trauma-hemorrhagic shock and prolonged hypotension. It was concluded that the mechanism responsible involved E2 since it decreased free iron levels (a potent inducer of oxidative stress) and decreased generation of mitochondrial reactive oxygen species which give rise to less oxidative stress during reperfusion.

The present observations of using L-NAME with estrogen concluded that serum creatinine in I/R after estrogen supplementation and L-NAME treatment (group V) increased significantly compared to estrogen-supplemented group (group VI) \((p<0.05)\). In addition, e-NOS gene expression in renal tissues decreased significantly compared to estrogen-supplemented group (group VI) whereas no significant difference was observed compared to I/R group (group II). These results revealed that L-NAME may blocke the protective role of estrogen in I/R injury thus emphasizing the concept that the action of estrogen is mediated through increase in NO synthesis by increasing e-NOS expression.

Vincent et al. \[34\] found that E2 increased cardiac levels of estrogen receptor (ER)-α, ER β, an ER-associated membrane protein caveolin-3, eNOS, and phosphorylated eNOS. This, exert potentially beneficial cardiovascular effects on NO. However, E2 also increased the cardiac levels of proteins associated with cardiovascular injury and inflammation including, AT1R, protein kinase C (PKC)\(_\beta\), phosphorylated PKC and phosphorylated extracellular signal regulated kinase (pERK)1/2, plasminogen activator inhibitor-1 (PAI-1), osteopontin and ED-1, and monocyte/macrophage-specific protein. E2 treatment led to similar effect on protein changes in hearts of L-NAME/AngII treated rats except that the increase in eNOS was prevented, on other hand, L-NAME/AngII and E2 had additive effects in increasing cardiac PKC\(_\beta\)S and PAI-1, thus yielding the highest levels of cardiac PAI-1 and PKCS.

In the present study rat tail systolic B.P. was significantly increased in estrogen supplementation and L-NAME treatment group (group V) compared to estrogen-supplemented (group IV), OX group (group III) and I/R group (group II) \((p<0.05)\). It is generally thought that inhibition of nitric oxide synthase leads to blood pressure elevation largely through reduction in vascular levels of the vasodilator nitric oxide. However, there are several reports suggesting that NO synthase inhibitors cause adrenal epinephrine release by both central and peripheral mechanisms \[25\].

This elevation in blood pressure caused by L-NAME may be by itself the cause of deterioration in the kidney condition and the change in e-NOS and p38 MAPK gene expression observed in this group. In patients with malignant hypertension, it was found, that the expression of e-NOS protein was decreased or diminished in the injured glomeruli, small renal arteries and arterioles, thus explained the cause that in spontaneously hypertensive stroke-prone (SP) rats receiving a high-salt/high-fat diet myocardial p38 MAPK was activated persistently during the development of cardiac hypertrophy and inactivated during decompensation \[3\].

Peroxynitrite level in renal tissues as a result of I/R increased in response to estrogen supplementation and L-NAME treatment (group V) significantly compared to estrogen-supplemented group (group VI) but no significant variation was recorded compared to I/R group (group II), suggesting that L-NAME can blocke the anti-oxidant effect of estrogen. The anti-oxidant effect of estrogen may be mediated partially by NO as the latter is an effective chain-breaking antioxidant in free radical-mediated lipid oxidation by reacting rapidly with peroxyl radicals \[8\].

In Conclusion, I/R injury produced renal dysfunction, which can be prevented by a protective role provided by estrogen against renal I/R injury.
as it produced an anti-oxidant effect mediated by increased expression of e-NOS and decreased peroxynitrite in renal tissue furthermore an E2 exerts anti-inflammatory effects manifested by decreased expression of renal p38 MAPK and L-NAME blocked this anti-oxidant effect of estrogen suggesting that this effect may be NO mediated.

References


