# Renoprotective effect of erythropoietin in rats subjected to ischemia/reperfusion injury: Gender differences

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***Background.*** *Renal ischemia reperfusion injury induces gender-dependent heat-shock protein 72 expression, which maintains membrane localization of renal $Na^+/K^+$ATPase-α1. The erythropoietin has a protecting effect against ischemia reperfusion injury in various organs. In this study, we investigated whether erythropoietin exerts a beneficial effect against post-ischemic renal injury. Furthermore, we studied the erythropoietin signaling on heat-shock protein 72 and $Na^+/K^+$ATPase-α1 expression and localization.*

***Methods.*** *In male and female Wistar rats, rHuEPO (1000 IU/bwkg intraperitoneal) or vehicle was administered 24 hours prior to unilateral left renal ischemia reperfusion (50 minutes). Kidneys were subsequently removed at hours 2 or 24 of the reperfusion; sham-operated rats served as controls (C) (n = 8/group). We measured serum erythropoietin, renal function, evaluated histological injury, and observed heat-shock protein 72 as well as $Na^+/K^+$ATPase-α1 protein level and localization. Additional groups were followed for 7-day survival.*

***Results.*** *Erythropoietin treatment was associated with better post-ischemic survival and less impaired renal function in males while diminishing the renal structural damage in both sexes. Endogenous erythropoietin was higher in males and increased in both genders after erythropoietin treatment. The erythropoietin treatment elevated protein levels of heat-shock protein 72 and $Na^+/K^+$ATPase-α1 in 24 hours in males, whereas in females, the already higher expression of heat-shock protein 72 and $Na^+/K^+$ATPase-α1 was not increased. Moreover, erythropoietin prevented ischemia reperfusion induced $Na^+/K^+$ATPase-α1 translocation from the basolaterale membrane in males.*

***Conclusion.*** *Erythropoietin diminishes gender difference in the susceptibility to renal post-ischemic injury and reduces post-ischemic structural damage while preserving kidney function, particularly in males. This additional protection may be associated with a heat-shock protein 72-mediated effect on $Na^+/K^+$ATPase-α1 expression and translocation. (Surgery 2011;150:39-47.)*

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Renal ischemia/reperfusion (I/R)-induced acute renal failure (ARF) still has high rates of morbidity and mortality. In renal transplantation, I/R is also a leading cause of delayed graft function and chronic allograft nephropathy.

The exact pathomechanism of the I/R is still unclear, but growing evidence indicates that sex differences exist in kidney response to renal ischemic injury.[1-3] Our previous studies have revealed the importance of a nitric oxide (NO) pathway, showing the pivotal role of NO and endothelin

in the gender-dependent renal response to ischemic injury[1] and aging.[4] Moreover, we also demonstrated the importance of $Na^+/K^+$ATPase-$\alpha$1 and heat-shock protein (HSP)72 in this gender-dependent injury.[1-3]

Erythropoietin (EPO) is an essential growth factor of hemopoietic progenitor cells,[5] but its extrahemopoietic effects imply additional therapeutic possibilities. Indeed, a wealth of experimental data is being generated with respect to the protective effect of EPO against the ischemic myocardium,[6,7] liver,[8,9] and renal injury.[10-12] Moreover, several clinical trials are also being processed. According to the trial of the Hannover Medical School (Hannover, Germany), rHuEPO alpha (administered 3 times after transplantation) significantly increased the glomerular filtration rate in transplant patients (ClinicalTrials.gov number: NCT00425698). The antiapoptotic, antioxidative, and anti-inflammatory effects of EPO have been investigated in ours and other laboratories,[12-16] but the complete molecular mechanism involved in the prevention of renal I/R injury is not yet fully understood.

A growing body of evidence supports the connection between EPO and the HSP70 family. It has been shown that EPO attenuates myocardial infarct size by enhancing the HSP70 protein level.[17] In addition, the induction of HSP70 by EPO administration inhibits apoptotic cell death in rat ischemic kidney.[18]

Previously, we demonstrated that renal I/R injury induces a gender-dependent HSP72 expression,[3] which maintains basolateral membrane localization of an essential tubular sodium transporter[19]—the $Na^+/K^+$ATPase. We also showed that, under ischemic conditions, its enzyme activity decreases in a gender- and time-dependent manner and, because of the disruption of the actin cytoskeleton, $Na^+/K^+$ATPase-$\alpha$1 internalizes or translocates from the basolateral to the apical membrane of renal tubular cells.[2]

Regarding the proven beneficial effects of EPO in renal ischemic injury, the aim of the present study was to investigate: (1) whether EPO treatment is protective against serious, unilateral, renal I/R damage; (2) whether EPO effect differs between female and male rats; and (3) the role of HSP72 and $Na^+/K^+$ATPase in this EPO effect.

## METHODS

**Animals.** Experiments were performed on sexually mature (7-week-old) female (weighing 182 ± 5 g) and male (weighing 182 ± 4 g) Wistar rats. All experimental protocols were in compliance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University of Budapest, Hungary. Rats were fed with a standard laboratory diet and water ad libitum.

**Experimental protocol.** rHuEPO beta (1000 IU/bwkg intraperitoneal [i.p.]; Roche, Budapest, Hungary) or vehicle was administered 24 hours prior to 50 minutes of the left renal ischemia injury to animals.[20,21]

General anesthesia was induced by i.p. administration of 50 mg $bwkg^{-1}$ pentobarbital sodium (Nembutal; Abbott Laboratories, Budapest, Hungary). Body temperature was maintained at 37°C on a heating pad throughout the anesthesia period. Renal ischemia was accomplished by cross-clamping the left renal artery and vein for 50 minutes with an atraumatic vascular clamp. Before the end of the period of ischemia, the contralateral kidney was removed after which the clamp was withdrawn, the abdomen closed, and the animals allowed to wake up.

In the first series, the survival of animals was followed for 7-days.

In the second series, rats later were reanaesthetized (50 mg $bwkg^{-1}$ i.p. Nembutal; Abbott Laboratories, Budapest, Hungary) allowing for the collection of blood samples from the abdominal aorta and the removal of kidneys at hours 2 (T2) and 24 (T24) of reperfusion ($n$ = 8/group). Uninephrectomized, sham-operated rats served as controls ($n$ = 8/group). Kidney samples were immediately snap-frozen in liquid nitrogen or fixed in 4% buffered formalin (pH 7.4) for future investigation.

**Labor parameters.** The serum EPO level was determined with EPO-DPC, an automated chemiluminescent immunoassay (Olympus Ltd., Budapest, Hungary), which detects both endogenous and exogenous EPO level.

Blood urea nitrogen (BUN) and serum creatinine levels were determined photometrically with commercially available kits (Diagnosticum Ltd., Budapest, Hungary) on a Hitachi-712 automated spectrophotometer.

**Renal histopathology.** The paraffin-embedded, 5-$\mu$m-wide sections of the excised kidney were stained with periodic acid–Schiff reagent. Samples were coded and semiquantitatively evaluated in blind tests by light microscopy, which were assessed by scoring widespread degeneration of tubular architecture, loss of brush borders, tubular dilation, swelling, vacuolization, and hyalinization within and outside of the tubular cells.

**Western blot analysis.** Protein determinations were performed in triplicate by Bradford analysis

using bovine serum albumin (Sigma Aldrich Co., Budapest, Hungary) as a standard. All reagents for Western blot were purchased from Sigma Aldrich Co. Samples (10 $\mu$g) were resolved electrophoretically on a 12.5% polyacrylamide gel and transferred to nitrocellulose membranes, which were blocked in Western buffer (20 mmol/L Tris, 150 mmol/L NaCl, 0.5% Tween20, 0.1% bovine serum albumin) for 1 hour at room temperature (RT). The membranes were incubated with HSP72 polyclonal antibodies (donated by Dr. Laszlo, Eotvos University, Budapest, Hungary)[4] and diluted to 1:9000 for 1 hour at RT. The membranes then were incubated in goat anti-rabbit IgG-HRP secondary antibody (sc 2004; BIO-Kasztel Ltd., Budapest, Hungary) diluted to 1:8000 for 30 minutes at RT. Additionally, membranes were incubated with a mouse monoclonal antibody against $Na^+/K^+$ATPase-$\alpha$1 subunit (sc 21712; BIO-Kasztel Ltd., Budapest, Hungary) diluted to 1:1000 for 1 hour at RT, and followed by goat antimouse IgG-HRP secondary antibody (sc 2005, BIO-Kasztel Ltd., Budapest, Hungary) diluted to 1:15000 for 30 minutes at RT. Blots were developed with enhanced chemiluminescence detection (AP-Biotech, Buckinghamshire, UK). Computerized densitometry of the specific bands was analyzed with Gel-Pro Analyzer 3.2 software (Media Cybernetics, Inc., Bethesda, MD). The values were normalized to b-actin and expressed as the relative optical density.

**Immunofluorescence staining.** Kidney samples were embedded in Shandon cryomatrix (Life Science Ltd., Budapest, Hungary) and cut to 5-$\mu$m-wide sections with a cryostat. They were incubated for 60 minutes at RT with the same antibody used in Western blotting, which were rabbit polyclonal antibodies against HSP72 diluted to 1:1000 and mouse monoclonal $Na^+/K^+$ATPase-$\alpha$1 diluted to 1:100. After repeated washing, slides were incubated with an Alexa Fluor 568 F $(ab')_2$ fragment of goat anti-rabbit IgG (A-11036; Csertex Ltd., Budapest, Hungary) and an Alexa Fluor 488 F $(ab')_2$ fragment of goat antimouse IgG (A-11017; Csertex Ltd., Budapest, Hungary) both were diluted to 1:100 for 30 minutes at RT. DNA was stained with Hoechst 33342 (Sigma-Aldrich Co., Budapest, Hungary) for 10 minutes at RT diluted to 1:1000. Appropriate controls were performed, omitting the primary antibodies to assure the specificity and to avoid autofluorescence. To visualize the stained tissues, a Zeiss LSM 510 Meta confocal laser-scanning microscope (Carl Zeiss, Jena, Germany) was applied.

**Statistical analysis.** The data were analyzed on STATISTICA 8 software (StatSoft Inc, Tulsa, OK) using factorial analysis of variance (ANOVA) for multiple comparisons followed by Fisher least significant difference post hoc test. Histological changes were analyzed using the Kruskal–Wallis test followed by multiple pair-wise comparisons according to the Fisher test. The cumulative proportion surviving analysis of significance was performed by Kaplan–Mayer analysis (log-rank test). The criterion for significance was $P < .05$ in all experiments. The data are presented as mean ± SD.

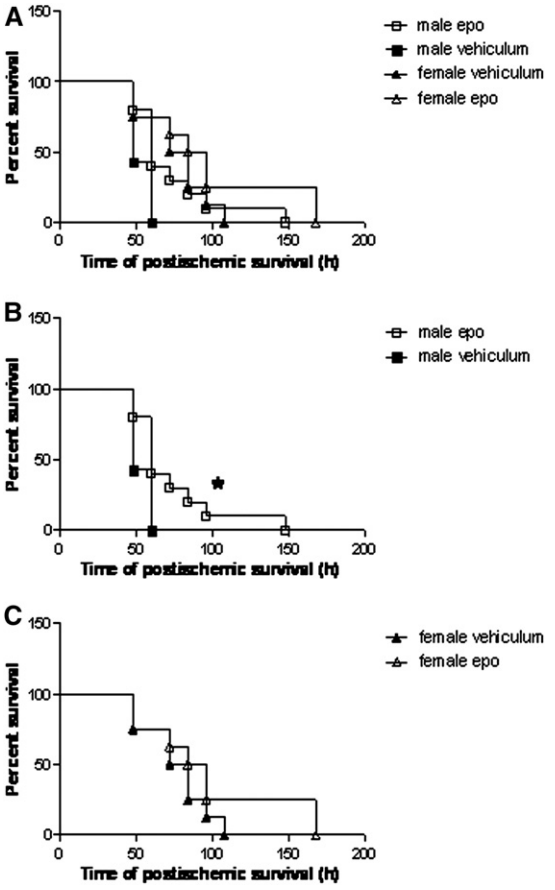


**Fig 1.** Effect of EPO treatment on the post-ischemic survival rate in female + vehicle (▲), female + EPO (△), male + vehicle (■), male + EPO (□) **(A)**; in male + vehicle (■) and male + EPO (□) **(B)**; in female + vehicle (▲) and female + EPO (△) **(C)** ($n = 8$). The cumulative proportion surviving analysis of significance was performed by Kaplan–Mayer analysis (log-rank test). *$P \leq .05$ vs male + vehicle.

## RESULTS

**Survival.** The EPO administration resulted in a remarkable amelioration of male's post-ischemic survival ($P \leq .05$). Although all untreated males died as a result of ARF by the third day, those treated with EPO survived until the sixth day, almost twice as long as those untreated. EPO resulted in

**Table I.** Serum levels of endogenous and exogenous EPO after renal I/R injury in female and male rats ($n$ = 8/group)*

| *Serum EPO (mIU/mL)* | *Control* | *T2* | *T24* |
|---|---|---|---|
| Female + vehicle | 7.7 ± 4.1 | 2.5 ± 0.8§ | 0.3 ± 0.6§,¶ |
| Male + vehicle | 11.9 ± 4.2† | 10.0 ± 2.4† | 1.4 ± 1.7†,§,¶ |
| Female + EPO | 15.2 ± 4.7‡ | 7.3 ± 3.1‡,§ | 1.4 ± 0.6‡,§,¶ |
| Male + EPO | 24.7 ± 15.0‡ | 25.7 ± 6.6†,‡ | 1.17 ± 0.3§,¶ |

*Values are means ± SD. Analysis of significance was performed by multiple comparison ANOVA followed by the Fisher post hoc test.
†$P < .05$ vs female.
‡$P < .05$ vs vehicle-treated.
§$P < .05$ vs control.
¶$P < .05$ vs T2.

**Table II.** BUN and serum creatinine levels after renal I/R injury in female and male rats with and without EPO treatment determined in serum samples from control and at 2 and 24 hours (T2 and T24, respectively) of reperfusion after 50 minutes of renal ischemia ($n$ = 8/group)*

| | *Control* | *T2* | *T24* |
|---|---|---|---|
| BUN (mmol/L) | | | |
| Female + vehicle | 4.6 ± 0.3 | 7.5 ± 0.73 | 36.2 ± 4.5§,¶ |
| Male + vehicle | 4.6 ± 0.3 | 10.2 ± 1 | 55.8 ± 10.2†,§,¶ |
| Female + EPO | 4.5 ± 0.9 | 8.4 ± 1.9 | 41.3 ± 7.0§,¶ |
| Male + EPO | 3.6 ± 0.5 | 6.3 ± 1.2 | 24.9 ± 4.5‡,†,§,¶ |
| Serum creatinine ($\mu$mol/L) | | | |
| Female + vehicle | 60.0 ± 5.6 | 94.8 ± 4.7 | 262 ± 24§,¶ |
| Male + vehicle | 53.0 ± 5.0 | 107 ± 7 | 368 ± 16†,§,¶ |
| Female + EPO | 50.5 ± 9.0 | 88.9 ± 18.0 | 324. ± 40‡,§,¶ |
| Male + EPO | 48.7 ± 4.1 | 84.7 ± 10.0 | 261 ± 50‡,†,§,¶ |

*Values are means ± SD. Analysis of significance was performed by multiple comparison ANOVA followed by the Fisher post hoc test.
†$P < .05$ vs female.
‡$P < .05$ vs vehicle-treated.
§$P < .05$ vs control.
¶$P < .05$ vs T2.

a slight improvement in females as well. To note, however, irrespective of the EPO administration, the 7-day survival was still better in females compared with their male counterparts (Fig 1).

**Serum EPO level.** The serum level of endogenous EPO was higher in males vs females not only in controls but post-ischemicly ($P < .05$; .001). EPO administration increased control and T2 EPO levels in both genders (untreated vs treated $P <$ .05; .01), whereas at T24, the effect of exogenous EPO treatment disappeared. The dynamics of post-ischemic changes in serum EPO levels followed a remarkably different manner between males and females; in males, the already higher EPO decreased only at T24, whereas in females, the EPO level dropped to a third of the control value already at T2 (Table I).

**BUN and serum creatinine levels.** Fifty minutes of ischemia resulted in an ARF as indicated by a progressive increase in renal function parameters in both sexes (control, T2 vs T24 $P < .001$; Table II). In males, the EPO treatment ameliorated the post-ischemic kidney failure indicated by lower BUN and creatinine levels (at T24 vehicle vs EPO-treated $P < .01$). EPO had no effect in females. Interestingly, at T24, the renal function parameters were even lower in EPO-treated males than in females with and without EPO treatment.

**Renal histopathology.** Kidneys from control rats showed normal kidney structure in all groups (Fig 2). After 50 min of ischemia, a progression was observed in the extent of tubular epithelial cell damage (loss of brush border, tubular dissolving, cell death, and loss of the nucleus integrity) as well as in the amount of hyaline casts until T24 (control vs T2 vs T24 in all groups $P < .05$; Table III). EPO ameliorated each evaluated parameters in both sexes without any gender difference (EPO vs vehicle treated $P < .05$). No kidney samples presented glomerular or relevant interstitial changes (not shown).

**HSP72 protein levels.** The HSP72 level was higher in untreated females than in male counterparts at every time point. After the ischemic insult,

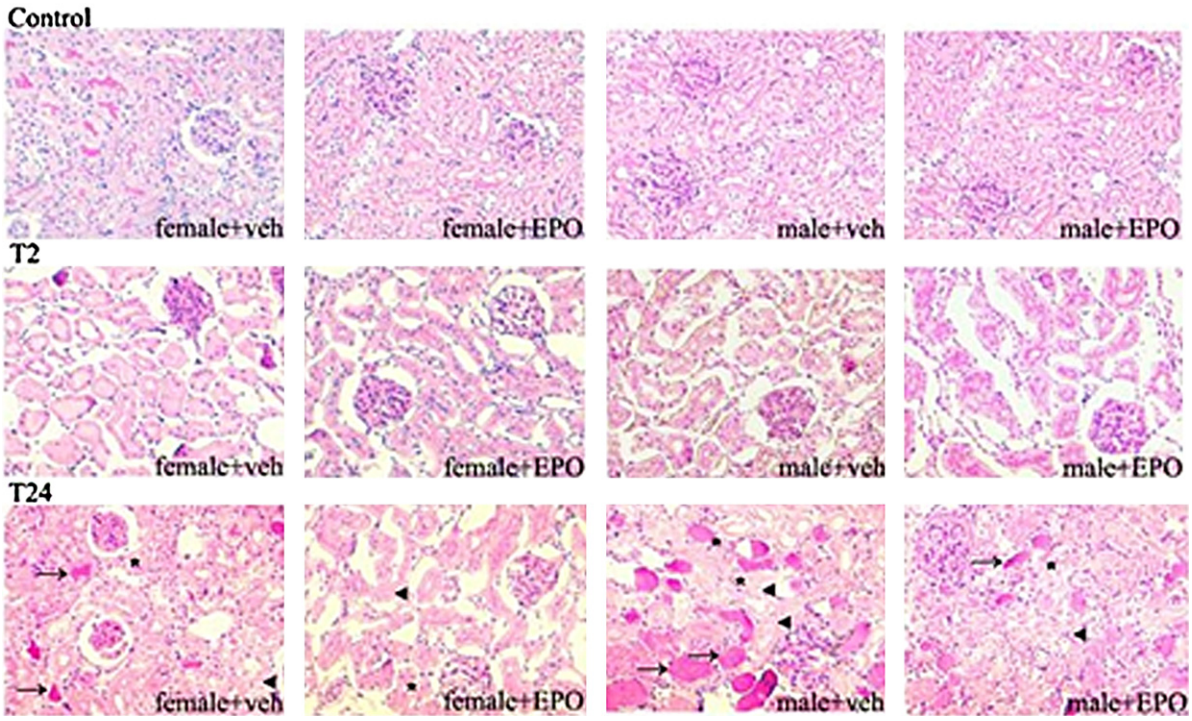


**Fig 2.** Representative pictures of histopathological changes in post-ischemic kidneys of female + vehicle, female + EPO, male + vehicle, and male + EPO rat. Histopathological changes in post-ischemic kidneys were determined and evaluated at a magnification of 40× by periodic acid-Schiff stained kidneys of control female + vehicle, control female + EPO, control male + vehicle, control male + EPO, T2 female + vehicle, T2 female + EPO, T2 male + vehicle, T2 male + EPO, T24 female + vehicle, T24 female + EPO, T24 male + vehicle, and T24 male + EPO. * loss of brush border; ◂ loss of nucleus integrity; → epithelial cast.

HSP72 protein levels increased (control vs T2 $P <$ .05) in a gender-dependent manner. In females, HSP72 reached its maximum at T2, whereas in males, the rate of increase was slower both with and without EPO treatment (female vs male $P <$ .05; .01; Fig 3). EPO increased the HSP72 protein level in males at T24, whereas in females, the already higher HSP72 level was not elevated (male vs female $P < .01$).

**$Na^+/K^+$ATPase-α1 subunit protein levels.** Similar to HSP72, the post-ischemic changes in $Na^+/K^+$ ATPase-α1 protein levels were different between the sexes. $Na^+/K^+$ATPase-α1 protein levels were higher in untreated females than in males at every time point ($P < .001$; .001; .05; Fig 4). EPO treatment was effective only in males by increasing the protein level at T24 (control and T2 vs T24 $P < .01$) to a level even higher than the EPO-treated females ($P < .01$).

**Immunolocalization of HSP72 and $Na^+/K^+$ATPase-α1 subunit.** Immunofluorescent staining was used to investigate the potential relationship between HSP72 and $Na^+/K^+$ATPase-α1 (Fig 5). In the tubules of control rats, $Na^+/K^+$ATPase-α1 was localized on the basolateral membrane domain of tubular cells, with minimal staining in the cytosol or the apical domain. No gender differences were observed at this time point. In contrast, HSP72 staining was virtually undetectable in the tubular cells of control rats. After ischemic injury, $Na^+/K^+$ATPase-α1 became more prominent in the cytosol compared with the controls, but this internalization was less significant in untreated females vs males. After EPO treatment, however, the $Na^+/K^+$ATPase-α1 localization remained more pronounced on the basolateral membrane in males as well.

## DISCUSSION

Ischemia-induced ARF is still an unsolved clinical problem with high morbidity and mortality. Moreover, ischemic injury during transplantation is a major cause of postoperative ARF and ultimately may result in the development of chronic allograft nephropathy.[22]

EPO previously has been suggested as a protective agent against post-ischemic injury in the central nervous system,[23] heart,[6] and liver.[8,9] Recently, EPO showed a beneficial effect in the kidney on post-ischemic survival[24] and renal structural

**Table III.** Histological evaluation of kidney damage in tissue samples from control and at 2 and 24 hours (control, T2 and T24, respectively) of reperfusion after 50 minutes of renal ischemia in female and male rats ($n$ = 8/group)*

| | *Control* | *T2* | *T24* |
|---|---|---|---|
| Tubular epithelial cell damage | | | |
| Female + vehicle | 0.0 (0–0) | 2.0 (2–2)‡ | 7.0 (7–7)‡,§ |
| Male + vehicle | 0.0 (0–0) | 2.0 (2–2)‡ | 7.0 (6–8)‡,§ |
| Female + EPO | 0.0 (0–1) | 2.5 (0–4)‡ | 5.0 (4–7)†,‡,§ |
| Male + EPO | 0.0 (0–1) | 2.5 (0–4)‡ | 5.0 (4–7)†,‡,§ |
| Epithelial casts | | | |
| Female + vehicle | 0.0 (0–0) | 0.0 (0–0) | 2.0 (2–2)‡,§ |
| Male + vehicle | 0.0 (0–0) | 0.0 (0–0) | 2.0 (2–2)‡,§ |
| Female + EPO | 0.0 (0–0) | 0.0 (0–0) | 0.5 (0–2)†,‡,§ |
| Male + EPO | 0.0 (0–0) | 0.0 (0–0) | 0.5 (0–2)†,‡,§ |

*Values are median ± range. Analysis of significance was performed by the Kruskal–Wallis test followed by multiple pairwise comparisons according to the Fisher test.
†$P < .05$ vs vehicle-treated.
‡$P < .05$ vs T0.
§$P < .05$ vs T2.

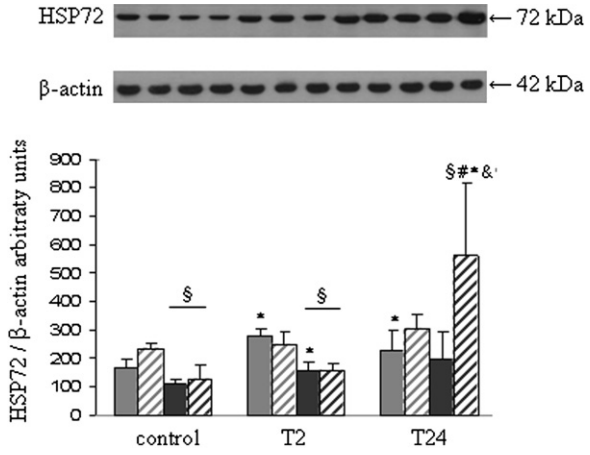


**Fig 3.** Effect of renal I/R injury on protein expression of HSP72 in female + vehicle, female + EPO, male + vehicle, and male + EPO rat kidney. Protein expression of HSP72 was determined in kidney samples from control and at T2 and T24 of reperfusion after 50 minutes of renal ischemia ($n$ = 8/group). Top: representative examples of Western blot analysis of HSP72 and beta-actin in kidney. Results for HSP72 protein levels in tissue samples were normalized to an internal standard. Female + vehicle (*gray*); female + EPO (*striped gray*); male + vehicle (*black*); male + EPO (*striped black*). Values are mean ± SD. §$P <$ .05 vs female, &$P < .05$ vs vehicle-treated, *$P < .05$ vs T0, #$P < .05$ vs T2. Analysis of significance was performed by multiple comparison ANOVA followed by the Fisher post hoc test.

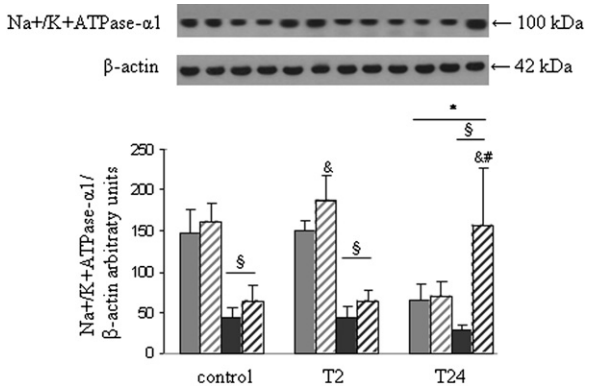


**Fig 4.** Effect of renal I/R injury on protein expression of $Na^+/K^+$ATPase-$\alpha$1 in female + vehicle, female + EPO, male + vehicle, and male + EPO rat kidney. Protein level of $Na^+/K^+$ATPase-$\alpha$1 was determined in kidney samples from control as well as at T2 and T24 of reperfusion after 50 minutes of renal ischemia ($n$ = 8/group). Top: representative examples of Western blot analysis of $Na^+/K^+$ATPase-$\alpha$1 and beta-actin in kidney. Results for $Na^+/K^+$ATPase-$\alpha$1 protein levels in tissue samples were normalized to an internal standard. Female + vehicle (*gray*); female + EPO (*striped gray*); male + vehicle (*black*); male + EPO (*striped black*). Values are mean ± SD. §$P <$ .05 vs female, &$P < .05$ vs vehicle treated, *$P < .05$ vs T0, #$P < .05$ vs T2. Analysis of significance was performed by multiple comparison ANOVA followed by the Fisher post hoc test.

damage.[25,26] Furthermore, in male rats, EPO treatment prevented the down-regulation of aquaporins and sodium transporters after 40 minute bilateral obstruction of renal arteries.[27] Conversely, in female rats, no EPO effect was revealed after myocardial infarction.[28] Nevertheless, no comparative studies have been done about the role of genders in regard to the protective effect of EPO against renal I/R.

We are the first to observe that the protective effect of EPO against renal I/R injury is sex related and more pronounced in male than in female rats.

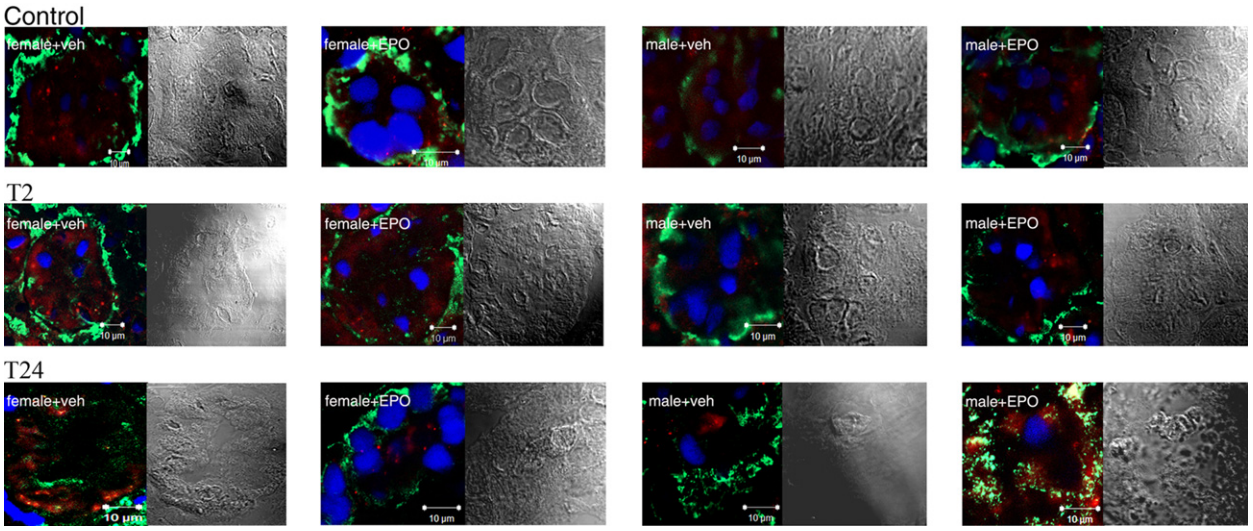


**Fig 5.** Localization of HSP72 (*red*) and $Na^+/K^+$ATPase-$\alpha$1 (*green*) in the tubule cells of representative samples using immunofluorescent staining followed by confocal laser scanning microscopy. The merged images show representative examples of co-localization (*yellow*) of HSP72 and $Na^+/K^+$ATPase-$\alpha$1 in kidney cortical sections of control female + vehicle, control female + EPO, control male + vehicle, control male + EPO, T2 female + vehicle, T2 female + EPO, T2 male + vehicle, T2 male + EPO, T24 female + vehicle, T24 female + EPO, T24 male + vehicle, T24 male + EPO rats. Nuclei are stained in blue. Scale bar: 10 $\mu$m. (Color version of figure is available online.)

Previously, we showed, however, that males are more vulnerable to renal I/R injury[1]; here, we demonstrated that EPO treatment in males prior to ischemia could prolong post-ischemic survival and its beneficial effect is more remarkable. Parallel with the survival, EPO improved the renal function and minimized the structural damage as well.

A significant sex difference was noted in EPO's beneficial effect on the aforementioned aspects, but we could not confirm this gender-disparity in post-ischemic kidney damage. Because morphological changes of the ARF develop roughly within 24 hours after the ischemic insult, one can hypothesize, that EPO administration might provide an opportunity for better recovery in a later phase.

In previous rodent and human studies, a higher level of EPO was demonstrated in males.[29,30] In line with these results, we also demonstrated a significant gender difference in EPO levels in control animals; moreover, our studies revealed remarkable sex difference after renal I/R injury. The exogenous EPO added to the already higher basal level in male rats resulted in an increased EPO concentration during and right after the ischemia in males. On the one hand, we assume that the higher level of EPO in males may provide an EPO-based, cell protective mechanism, which is less pronounced in females. On the other hand, because women on hemodialysis therapy require a greater dose of EPO to attain a hematocrit equivalent with men[31] and because they show a lower level of EPO,[29,30] one can postulate that in our study a higher exogenous EPO also would be needed to reach the same protective effect that we found in males; however, to evaluate this problem, additional studies would be needed, and in the latter case, one also should expect a higher rate of adverse effects.

We propose that EPO develops its protective effect in males partially through the increased HSP72 level, which promotes the protection of $Na^+/K^+$ATPase-$\alpha$1. Here, we demonstrated that EPO in males induced significant HPS72 elevation at 24 hours of reperfusion. Our results are in concordance with previous data showing that rHuEPO increases HSP72 levels with a maximal effect in hour 24 of reperfusion preventing renal damage after I/R injury.[18] EPO might exert its increasing effect on HSP72 through the janus kinase (JAK) and signal transducer and activator of transcription (STAT), which are known not only as members of EPO's downstream signal pathway[32,33] but also as activators of HSP72.[34] Also supporting this idea is the study, which demonstrated, in liver, that the STAT5 pathway is 10-fold more active in males than in females.[35] Thus, it is also conceivable that EPO would protect against I/R injury because of this JAK/STAT/HSP72 pathway.

The post-ischemic decrease in the expression, function, and internalization of $Na^+/K^+$ATPase-$\alpha$1

is a well-established feature.[36,37] Parallel to this internalization, HSP72 moves into the cytoskeletal fraction of tubular cells, advancing the reintegration of cytoskeleton, which is essential for the appropriate localization and function of the $Na^+/K^+$ATPase-$\alpha$1.[38,39,40] In the present study, 24 hours after I/R injury, the $Na^+/K^+$ATPase-$\alpha$1 protein level was noticeably preserved in EPO-treated males. Furthermore, post-ischemic internalization and translocation of the enzyme also were prevented by EPO in a gender-dependent manner; male rats displayed a significantly more robust response to EPO than females. Parallel to this phenomenon, HSP72 translocated toward the plasma membrane where it partially co-localized with $Na^+/K^+$ATPase-$\alpha$1 in the post-ischemic period. This co-localization was the most prominent in the EPO-treated males at T24 and contrasted sharply with the primarily cytoplasmic localization of HSP72 in all control animals. Thus, one can speculate that EPO, through the enhancement of HSP72, helps to preserve the integrity of tubular cells and increases the protein level and stability of the $Na^+/K^+$ATPase-$\alpha$1.

These observations are in accordance with our previous results, stating that HSP72 participates in the stabilization of the cytoskeleton as well as in the preservation of both the localization and the function of $Na^+/K^+$ATPase-$\alpha$1.[3] According to the existing data, HSP72 associates with other chaperons (HSP25 and HSP90) to stabilize the cytoskeletal anchorage and the re-compartmentalization of $Na^+/K^+$ATPase-$\alpha$1.[39,40] In an in vitro study, the close relation between HSP72 and $Na^+/K^+$ATPase-$\alpha$1 also was proven by immunoprecipitation showing that the binding of HSP70 to $Na^+/K^+$ATPase-$\alpha$1 is dynamic and specific.[41]

Taken together, we might speculate that enhanced levels of HSP72, after EPO treatment, can stabilize the anchorage of the $Na^+/K^+$ATPase-$\alpha$1 to the cytoskeleton and restore the structure of the aggregated enzyme, which might be an important pathway in the protective effect of EPO in male rats.

In summary, our results based on both survival and molecular studies suggest that EPO protects against severe, unilateral renal I/R injury, especially in male rats. Furthermore, we believe this beneficial effect partly might be the result of EPO's HSP72-mediated impact on $Na^+/K^+$ATPase-$\alpha$1. With respect to the future, the effective and safe administration of EPO and EPO mimetic drugs also may have therapeutic potential in preventing ischemic kidney injury in clinical settings such as open-heart, aorta surgery, and renal transplantations. Finally, the individualized EPO administration between males and females also might be considered in everyday clinical practice.

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## REFERENCES

1. Müller V, Losonczy G, Heemann U, Vannay A, Fekete A, Reusz G, et al. Sexual dimorphism in renal ischemia-reperfusion injury in rats: possible role of endothelin. Kidney Int 2002;62:1364-71.
2. Fekete A, Vannay A, Vér A, Vásárhelyi B, Müller V, Ouyang N, et al. Sex differences in the alterations of Na(+), K(+)-ATPase following ischemia-reperfusion injury in the rat kidney. J Physiol 2004;555:471-80.
3. Fekete A, Vannay A, Vér A, Rusai K, Müller V, Reusz G, et al. Sex differences in heat shock protein 72 expression and localization in rats following renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 2006;291:806-11.
4. Erdely A, Greenfeld Z, Wagner L, Baylis C. Sexual dimorphism in the aging kidney: effects on injury and nitric oxide system. Kidney Int 2003;63:1021-6.
5. Koury MJ, Bondurant MC. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 1990;248:378-81.
6. Calvillo L, Latini R, Kajstura J, Leri A, Anversa P, Ghezzi P, et al. Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. Proc Natl Acad Sci 2003;100:4802-6.
7. Tang YD, Hasan F, Giordano FJ, Pfau S, Rinder HM, Katz SD. Effects of recombinant human erythropoietin on platelet activation in acute myocardial infarction: results of a double-blind, placebo-controlled, randomized trial. Am Heart J 2009;158:941-7.
8. Yilmaz S, Ates E, Tokyol C, Pehlivan T, Erkasap S, Koken T. The protective effect of erythropoietin on ischemia/reperfusion injury of liver. HPB (Oxford) 2004;6:169-73.
9. Schmeding M, Rademacher S, Boas-Knoop S, Roecken C, Lendeckel U, Neuhaus P, et al. rHuEPo reduces ischemia-reperfusion injury and improves survival after transplantation of fatty livers in rats. Transplantation 2010;27:161-8.
10. Chatterjee PK. Novel pharmacological approaches to the treatment of renal ischemia-reperfusion injury: a comprehensive review. Naunyn Schmiedebergs Arch Pharmacol 2007;376:1-43.
11. Vesey DA, Cheung C, Pat B, Endre Z, Gobé G, Johnson DW. Erythropoietin protects against ischaemic acute renal injury. Nephrol Dial Transplant 2004;19:348-55.
12. Rusai K, Prókai A, Szebeni B, Fekete A, Treszl A, Vannay A, et al. Role of serum and glucocorticoid-regulated kinase-1 in the protective effects of erythropoietin during renal ischemia/reperfusion injury. Biochem Pharmacol 2010;79:1173-81.
13. Bonventre JV. Mechanisms of ischemic acute renal failure. Kidney Int 1993;43:1160-78.
14. Paller MS. The cell biology of reperfusion injury in the kidney. J Investig Med 1994;42:632-9.
15. Weight SC, Bell PR, Nicholson ML. Renal ischemia-reperfusion injury. Br J Surg 2004;83:162-70.
16. Versteilen AM, Di Maggio F, Leemreis JR, Groeneveld AB, Musters RJ, Sipkema P. Molecular mechanisms of acute renal failure following ischemia/reperfusion. Int J Artif Organs 2004;27:1019-29.

17. Xu B, Dong GH, Liu H, Wang YQ, Wu HW, Jing H. Recombinant human erythropoietin pretreatment attenuates myocardial infarct size: a possible mechanism involves heat shock Protein 70 and attenuation of nuclear factor-kappaB. Ann Clin Lab Sci 2005;35:161-8.
18. Yang CW, Li C, Jung JY, Shin SJ, Choi BS, Lim SW, et al. Preconditioning with erythropoietin protects against subsequent ischemia-reperfusion injury in rat kidney. FASEB J 2003;17:1754-5.
19. Skou JC, Esmann M. The Na, K-ATPase. J Bioenerg Biomembr 1992;24:249-61.
20. Patel NS, Sharples EJ, Cuzzocrea S, Chatterjee PK, Britti D, Yaqoob MM, et al. Pretreatment with EPO reduces the injury and dysfunction caused by ischemia/reperfusion in the mouse kidney in vivo. Kidney Int 2004;66:983-9.
21. Kiris I, Kapan S, Kilbas A, Yilmaz N, Altuntaş I, Karahan N, et al. The protective effect of erythropoietin on renal injury induced by abdominal aortic-ischemia-reperfusion in rats. J Surg Res 2008;149:206-13.
22. Perico N, Cattaneo D, Sayegh MH, Remuzzi G. Delayed graft function in kidney transplantation. Lancet 2004;364: 1814-27.
23. Sakanaka M, Wen TC, Matsuda S, Masuda S, Morishita E, Nagao M, et al. In vivo evidence that erythropoietin protects neurons from ischemic damage. Proc Natl Acad Sci 1998; 95:4635-40.
24. Nemoto T, Yokota N, Keane WF, Rabb H. Recombinant erythropoietin rapidly treats anemia in ischemic acute renal failure. Kidney Int 2001;59:246-51.
25. Johnson DW, Pat B, Vesey DA, Guan Z, Endre Z, Gobe GC. Erythropoietin protects against ischaemic acute renal injury. Nephrol Dial Transplant 2004;19:348-55.
26. Sharples EJ, Patel N, Brown P, Stewart K, Mota-Philipe H, Sheaff M, et al. Erythropoietin protects the kidney against the injury and dysfunction caused by ischemia-reperfusion. J Am Soc Nephrol 2004;15:2115-24.
27. Gong H, Wang W, Kwon TH, Jonassen T, Li C, Ring T, et al. EPO and alpha-MSH prevent ischemia/reperfusion-induced down-regulation of AQPs and sodium transporters in rat kidney. Kidney Int 2004;66:683-95.
28. Hale SL, Sesti C, Kloner RA. Administration of erythropoietin fails to improve long-term healing or cardiac function after myocardial infarction in the rat. J Cardiovasc Pharmacol 2005;46:211-5.
29. Kumagai J, Yorioka N, Kawanishi H, Moriishi M, Komiya Y, Asakimori Y, et al. Relationship between erythropoietin and chronic heart failure in patients on chronic hemodialysis. J Am Soc Nephrol 1999;10:2407-11.
30. Pequignot JM, Spielvogel H, Caceres E, Rodriguez A, Semporé B, Pequignot J, et al. Influence of gender and endogenous sex steroids on catecholaminergic structures involved in physiological adaptation to hypoxia. Pflugers Arch 1997;433:580-6.
31. Ifudu O, Uribarri J, Rajwani I, Vlacich V, Reydel K, Delosreyes G, et al. Gender modulates responsiveness to recombinant erythropoietin. Am J Kidney Dis 2001;38:518-22.
32. Breggia AC, Wojchowski DM, Himmelfarb J. JAK2/Y343/STAT5 signaling axis is required for erythropoietin-mediated protection against ischemic injury in primary renal tubular epithelial cells. Am J Physiol Renal Physiol 2008;295:1689-95.
33. Tsuji-Takayama K, Otani T, Inoue T, Nakamura S, Motoda R, Kibata M, et al. Erythropoietin induces sustained phosphorylation of STAT5 in primitive but not definitive erythrocytes generated from mouse embryonic stem cells. Exp Hematol 2006;34:1323-32.
34. Madamanchi NR, Li S, Patterson C, Runge MS. Reactive oxygen species regulate heat-shock protein 70 via the JAK/STAT pathway. Arterioscler Thromb Vasc Biol 2001;21:321-6.
35. Choi HK, Waxman DJ. Plasma growth hormone pulse activation of hepatic JAK-STAT5 signaling: developmental regulation and role in male-specific liver gene expression. Endocrinology 2000;141:3245-55.
36. Molitoris BA, Dahl R, Geerdes A. Cytoskeleton disruption and apical redistribution of proximal tubule Na(+)-K(+)-ATPase during ischemia. Am J Physiol Renal Physiol 1992; 263:488-95.
37. Kwon TH, Frøkiaer J, Han JS, Knepper MA, Nielsen S. Decreased abundance of major Na(+) transporters in kidneys of rats with ischemia-induced acute renal failure. Am J Physiol Renal Physiol 2000;278:925-39.
38. Aufricht C, Lu E, Thulin G, Kashgarian M, Siegel NJ, Van Why SK. ATP releases HSP-72 from protein aggregates after renal ischemia. Am J Physiol Renal Physiol 1998;274:268-74.
39. Bidmon B, Endemann M, Müller T, Arbeiter K, Herkner K, Aufricht C. HSP-25 and HSP-90 stabilize Na, K-ATPase in cytoskeletal fractions of ischemic rat renal cortex. Kidney Int 2002;62:1620-7.
40. Riordan M, Garg V, Thulin G, Kashgarian M, Siegel NJ. Differential inhibition of HSP72 and HSP25 produces profound impairment of cellular integrity. J Am Soc Nephrol 2004;15:1557-66.
41. Riordan M, Sreedharan R, Wang S, Thulin G, Mann A, Stankewich M, et al. HSP70 binding modulates detachment of Na-K-ATPase following energy deprivation in renal epithelial cells. Am J Physiol Renal Physiol 2005;288:1236-42.