Erythropoietin Attenuates Apoptosis After Ischemia-Reperfusion–Induced Renal Injury in Transiently Hyperglycemic Wister Rats

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ABSTRACT

Background. Hyperglycemia is associated with a decreased tolerance to ischemia and an increased severity of renal ischemia reperfusion (I/R) injury. It has been suggested that erythropoietin (EPO) attenuates this effect in normoglycemic animals. This study sought to examine the effects of EPO on treatment renal I/R injury (IRI) in transiently hyperglycemic rats.

Material and Methods. Twenty-eight male Wister rats anesthetized with isoflurane received glucose (2.5 $g.kg^{-1}$ intraperitoneally) before right nephrectomy. They were randomly assigned to four groups: sham operation (S); IRI (ISO); IRI+EPO, (600 UI kg^{-1} low-dose EPO [EL]); and IRI+EPO 5000 UI kg^{-1} (high-dose EPO [EH]). IRI was induced by a 25-minute period of left renal ischemia followed by reperfusion for 24 hours. Serum Creatinine and glucose levels were measure at baseline (M1), immediately after the ischemic period (M2), and at 24 hours after reperfusion (M3). After sacrificing the animals, left kidney specimens were submitted for histological analysis including flow cytometry to estimate tubular necrosis and the percentages of apoptotic, dead or intact cells.

Results. Scr in the ISO group was significantly higher at M3 than among the other groups. Percentages of early apoptotic cells in ISO group were significantly higher than the other groups. Percentages of late apoptotic cells in S and ISO groups were significantly greater than EL and EH groups. However, no significant intergroup differences were observed regarding the incidence of tubular necrosis.

Conclusions. Our results suggested that, although not preventing the occurrence of tubular necrosis, EPO attenuated apoptosis and glomerular functional impairment among transiently hyperglycemic rats undergoing an ischemia/reperfusion insult.

Ischemia reperfusion (I/R) causes acute renal injury, which is a leading cause of delayed graft dysfunction and chronic allograft nephropathy after renal transplantation.[1]

Hyperglycemia is associated with a decreased tolerance to ischemia and an increased severity of the renal I/R injury.[2]

Transient hyperglycemia which is commonly found even in non-diabetic patients after renal transplantation,[3,4] may accentuate the ischemic renal injury, apoptosis, and antigenic inflammatory responses which increase the risk of graft rejection.[4]

Erythropoietin (EPO) administered before renal I/R may exert renoprotective effects in normoglycemic animals.[5] However, the effect has not been widely studied in transiently hyperglycemic animals. We hypothesized that EPO also exerted renoprotective effects in transiently hypoglycemic rats undergoing renal I/R.

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METHODS

The Institutional Review Board on Animal Experimentation approved the study. Twenty-eight male Wister rats[6] weighing more than 250 g were assigned to four groups, according to a random; electronically generated group S (n = 6) underwent sham procedures; group ISO (n = 6) to single-kidney I/R, as described elsewhere;[7] group EL (n = 8), low-dose EPO (600 UI.kg^{-1} intravenously) followed by single-kidney; I/R; and group EH (n = 8), high-dose erythropoietin (5000 UI.kg^{-1} intravenously) followed by I/R. EPO (Eprex) used in this study was purchased from Janssen Cilag Pharmaceutical Ltd. (São Paulo, Brazil).

Isoflurane was used for Induction of anesthesia followed by tracheal intubation. Lungs were mechanically expended through a rodent ventilator (Harvard Rodent Ventilator 683, South Natick, MA) that was set to deliver tidal volumes of 10 mL kg^{-1} and a respiratory rate of 80 breaths per minute. Further adjustments were made to maintain normocarbia. Oxygen (100%) and isoflurane (1.5%) were administered throughout the procedure. End-tidal carbon dioxide partial pressure, and inspired and expired anesthetic gas concentrations were continously measured throughout the procedure (Datex Engstron, Finland). Mean arterial pressure (MAP) was electronically and continuously recorded through a catheter surgically inserted into the left carotid artery (Datex Engstron). The right internal jugular vein was cannulated for fluid and drug administration as well as central venous pressure measurements. Rectal temperature was maintained in the 36°C to 38°C range.[8] Glucose (2.5 g kg^{-1}) was administered intraperitoneally after tracheal intubation. Lactated Ringer's solution (3 mL min^{-1}) was administered intravenously through a Harvard pump 22 (Instech, Plymouth Meeting, PA).

Each subject understand a midline laparotomy followed by a right nephrectomy. EPO was administered intravenously to rats assigned to groups EL (600 UI kg^{-1}) and EH (5000 UI kg^{-1}), which corresponded to human equivalent doses of 96 UI kg^{-1}and 800 UI kg^{-1}, respectively.[9] Saline was administered to rats allocated to the ISO group. Animals assigned to the sham group underwent the same procedures, except for the renal ischemia. Surgical wounds were infiltrated with 0.25% bupivacaine at the end of the procedure. The inhalation anesthetic was discontinued and hosts were allowed to recover spontaneously. After tracheal extubation, animals were moved to heated individual cages. Free access to water and food was granted postoperatively. Arterial blood samples for measurements of serum creatinine (Scr) and glucose (Sglu) levels were drawn after carotid artery cannulation (M1), after kidney reperfusion (M2), and 24 hours there after (M3). Rats were then anesthetized, sacrificed, and the left kidney excised and sectioned longitudinally into two fragments for histological analyses. One fragment was fixed in Duboscq Brazil's solution (formaldehyde, picric acid, acetic acid, and absolute ethyl alcohol), embedded in paraffin, stained with hematoxylin- eosin, and examined under optical microscopy by a pathologist unaware of the groups to from which the specimen was derived. Cell damage scores were attributed to each specimen, according to the following classification, based on the percentage of necrotic tubular cells in the specimen:0 (no necrosis); 1 (mild injury), less than 10%; 2 (moderate), 11% to 25%; 3 (moderately severe), 26% to 50%; 4 (severe), 51% to 75%; or 5 (very severe), when more than 75% of examined tubular cells necrotic.[10] The remaining fragment was subjected to flow cytometry (FCM),[11–14] to measure the percentages of early apoptotic, late apoptotic, dead, and intact cells. Analyses were performed on a BD FACSCalibur™ flow cytometer (Becton, Dickinson & Co., San Jose, CA). Normally distributed variables (MAP, Scr, analysis of vanance and Sglu) were submitted to two-way (groups × measuring occasions interactions) repeated measures analysis of variance (ANOVA); followed by Student-Newman-Kuels post hoc tests. Kruskal-Wallis tests were applied for intergroup comparisons of histologically and FCM-derived data. The significance level was set at $\alpha = 0.05$.

Table 1. Intergroup Comparisons of Mean Arterial Pressure at the Measurement Occasions

	Measurement Occasions		
Groups	M1	M2	M3
S	86.1 ± 27.4	97.0 ± 14.9	93.0 ± 21.5
ISO	94.7 ± 20.0	89.7 ± 17.4	90.2 ± 17.9
EH	103.8 ± 9.4	100.4 ± 15.9	92.1 ± 13.2
EL	102.6 ± 19.6	82.4 ± 21.5	84.8 ± 24.1

Abbreviations: S, sham; ISO, single-kidney ischemia-reperfusion; EH, high-dose erythropoietin (5000 UI kg1 intravenously) followeed by I/R; EL, low-dose erythropoietin (600 UI - 1 intravenously) followed by ISO; M1, after carotid artery cannulation; M2, after kidney reperfusion; M3, 24 hours after reperfusions.

Table 2. Intergroup Comparisons of Serum Glucose Levels at the Measurement Occasions

	Measurement Occasions		
Groups	M1	M2	M3
S	212.3 ± 48.4	302.5 ± 80.0*	181.8 ± 12.7
ISO	241.2 ± 36.0	348.3 ± 52.3*	135.5 ± 47.6†
EH	174.9 ± 30.5	271.9 ± 95.2*	166.4 ± 26.2
EL	190.8 ± 30.3	282.0 ± 85.4*	163.0 ± 31.1

Abbreviations: S, sham; ISO, single-kidney-HR ischemia-reperfusion (I/R); EH, high-dose enythropoietin (5000 UI.kg-1 inravenously) followed by I/R; EL, low-dose erythropoietin (600 UI.kg−1 intravenously) followed by single-kidney I/R: M1, after carotid artery cannulation; M2, after kidney reperfusion; M3, 24 hours after reperfusion.

*Significant increases in glucose levels occurred in all groups at M2 ($P < .01$).

†$P < .01$ compared to the other groups, at M3.

RESULTS

The MAP values did not differ among the groups at the measurement occasions ($F_{(6.54)} = 1.83$; $P = .1$; Table 1).

Although there were no significant group × measurement occasion interaction effects ($F_{(6,48)} = 2.04$; $P = .07$) Sglu levels were increased in all groups at M2. At M3, the

Table 3. Intergroup Comparisons of Serum Creatinine Levels at the Measurement Occasions

	Measurement Occasions		
Groups	M1	M2	M3
S	0.3 ± 0.1	0.4 ± 0.2	0.7 ± 0.2*
ISO	0.4 ± 0.0	0.7 ± 0.1	4.4 ± 0.9*,†
EH	0.3 ± 0.0	0.6 ± 0.1	2.7 ± 0.8*,‡
EL	0.3 ± 0.0	0.6 ± 0.1	2.5 ± 0.7*,‡

Abbreviations: S, sham ISO, single-kidney ischemia-reperfusion (I/R); EH, high-dose erythropoietin (5000 UI.kg−1 intravenously) followed by I/R; EL, low-dose erythropoietin (600 UI.kg−1 intravenously) followed by single-kidney I/R; M1, after carotid artery cannulation: M2, after kidney reperfusion; M3, 24 hours after reperfusion.

*Significant increases in glucose levels occurred in all groups at M3 ($P < .01$).

†$P < .01$ compared to groups S, E, and EH at M3.

‡$P < .01$ compared to group S at M3.

Table 4. Percentages of Tubular Cells in Specimens of the Study Groups, According to Flow Cytometric Classes

Classes	S	ISO	EH	EL
Early apoptotic	4.9 [2.5; 6.9]	48.2† 5.7 [29.5; 61.7]†	1.4 [0.4; 3.0]	1.5 [1.3; 3.4]
Late apoptotic	1.2† [0.7; 1.9]	5.7† [2.9; 7.6]	0.1 [0.1; 0.2]	0.1 [0.0; 0.1]
Dead	3.9 [0.7; 10.0]	4.7 [0.8; 14.3]	0.8 [0.6; 0.9]	0.4 [0.2; 0.7]
Intact	85.8 [82.7; 91.4]	45.2† [31.6; 50.1]	97.7 [95.8; 98.7]	97.8 [97.0; 98.1]

Abbreviations; S, sham; ISO, single-kidney ischemia-reperfusion (I/R); EH, high-dose erythropoietin (5000 UI.kg−1 intravenously) followed by I/R: EL, low-dose erythropoietin (600 UI.kg−1, intravenously) followed by single-kidney I/R.
*Expressed as median E [25th percentile; 75th percentile].
†$P < .01$ compared to the other groups.

ISO group showed significantly lower glucose levels, (Table 2).

Significant group × measurement occasion interaction effects were observed regarding Scr levels ($F_{(6,50)} = 26.46$; $P < .01$). No intergroup differences were found at M1 or M2. The ISO group exhibited significantly greater values compared with the other groups at M3. No significant differences were observed between EH and EL groups at M3, although, the Scr levels of these groups were significantly higher than those of the S group (Table 3).

Cell damage scores (median [25th; 75th percentiles]) of kidneys from the S group [0 (0;0)] were significantly lower than those of the EH (4.0 [4.0; 4.0]); EL (4.5 [4.0; 5.0]) and ISO (5.0 [3.5; 5.0]) groups ($P < .001$).

The percentage of early apoptotic cells in specimens from the ISO group were significantly higher than those of the other groups. Percentages of late apoptotic cells in specimens from S and ISO groups were significantly higher than those obtained from EH and EL groups. No significant differences were noted regarding the percentages of dead cells in the specimens among the groups. The percentages of intact cells in specimens from S, EH, and EL groups were significantly greater higher than those found in the ISO group (Table 4).

DISCUSSION

The main finding of this study was significants attenuation of renal tubular cells apoptosis following administration of EPO before the induction of I/R in rats made hyperglycemic by intraperitoneal administration of glucose. This observation may be a consequence of the inhibition of caspases, especially caspase 3. Which mediates apoptosis in hyperglycemic environment.[15]

This effect was accompanied by glomerular functional loss as the Scr levels were significantly lower among rats receiving EPO. It has been shown that EPO inhibits the expression of inflammatory cytokines interleukin-10 and tumor necrosis factor–α,[16] which may explain our findings.

This study confirmed other findings that hyperglycemia itself is assoicated with renal injury,[17] as Rats-undergoing sham procedures exhibited significantly higher percentages of late apoptotic tubular cells.

We also observed that cell necrosis was not affected by erytpetin administration, suggesting that although decreasing the deleterious effects on tubular cells, of I/R the EPO dosages of used in this study were not sufficient to prevent tubular necrosis. Given the small number of animals in each group, our study may have been underpowered to detect the existence of small differences.

Another interesting finding was that Sgl levels at 24 hours after reperfusion were significantly lower in among animals that did not receive EPO, but underwent renal ischemia (group ISO). Increased glucose and amino acid excretion and decreased sodium reabsorption are known to be associated with renal cell injury and decreased glomerular function,[18] among animals experiencing renal ischemia. Because no difference in Sglu levels was shown among the other groups, this finding may provide additional evidence for the protective effects of EPO administered before a renal ischemic insult.

It has been shown that the synthesis of EPO is inhibited by transient hyperglycemia associated with kidney I/R injury.[19] Although we did not measure blood levels of EPO in this study, we speculate that the exogenously administered hormone may have compensated to some extent for the decreased endogenous EPO levels.

Neutrophil infiltration and activation have been assoicated with I/R-induced tubular cell injury in transiently hyperglycemic rats. D-ribose administered before induction of renal ischemia attenuates this effect,[20] suggesting several mechanisms are involved in I/R-induced tubular cell damage in hyperglycemic rats. Thus, the concomitant use of multiple pharmacological interventions may produce more pronounced protective effects, an hypothesis that deserves further study.

We concluded that EPO attenuated apoptosis and impairment of glomerular function but not tubular necrosis, in transiently hyperglycemic rats undergoing an to I/R insult.

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