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Attenuation of pro-inflammatory cytokines and oxidative stress by misoprostol in renal ischemia/reperfusion in rats

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The ischemia/reperfusion (I/R) process alters metabolic pathways, releasing reactive oxygen species and pro-inflammatory cytokines that cause tissue necrosis and activate cellular apoptotic pathways. Misoprostol (MSP) is a prostaglandin E1 analog that has demonstrated a cytoprotective role in the I/R process. The study objective was to evaluate the effects of MSP on the regulation of pro-inflammatory and oxidative stress mediators in an I/R-induced acute kidney injury rat model. Wistar rats were divided into 3 groups. Sham and I/R were given 1 mL/day of physiological solution; MSP+I/R was given intragastric MSP (300 μg/kg) for 3 days. For I/R and MSP+IR, the renal hilum was clamped for 45 min, followed by 15 h of reperfusion. Renal function tests, pro-inflammatory cytokines, mediators of oxidative stress, and histological analysis were evaluated. Pro-inflammatory cytokine activity was significantly attenuated in the MSP+I/R group. However, there was no statistically significant difference between Sham and MSP. Regarding antioxidant activity, MSP+I/R showed a significant decrease in these mediators compared with Sham and I/R. Histologically, scarce medullary necrosis was observed with a preserved renal cortex in the MSP group.

1. Introduction

Ischemia/reperfusion (I/R) injury usually causes acute renal failure and remains a discussion topic in kidney transplantation, representing a high morbidity and mortality in medical practice. Improving the kidney’s tolerance to this process and avoiding functional decrease are significant issues (Zou et al. 2013).

The ischemia process represents a series of events occurring during the transitory blockage of blood and oxygen flow, which causes a deficit in intracellular adenosine triphosphate and electrolyte imbalance in the cell membrane. This creates a destabilization of the cellular structure and simultaneously generates oxidative stress and reactive oxygen species (ROS), which are reinforced by a hypoxia-related decrease in antioxidant enzyme activity, such as superoxide dismutase (SOD), catalase, and glutathione. Free radicals like superoxide that cause lipid peroxidation are also generated; this oxidation results in production of malondialdehyde (MDA), hydroxynonenal, and hexanal, causing significant damage to cellular function. Subsequent tissue reperfusion causes an increase in oxygen levels, further increasing ROS production, decreasing antioxidant cell capacity, and increasing lipid peroxidation, which causes protein denaturation, DNA breakdown, apoptosis, and functional loss (Chatauret et al. 2014; Muñiz et al. 2014). Furthermore, an overlapped cytokine liberation triggers an amplified inflammatory reaction, leading to progressive damage or irreversible tissue deterioration and fibrosis (Nath and Norby 2000; Troncoso et al. 1995; Parra et al. 2010; Ponticelli et al. 2014).

To minimize these effects of the I/R process, different drug types have been used, among which are the prostaglandin E1 analogs (PGE1), known for their vasodilator, antiplatelet, and fibrinolytic effects and for modulating cell proliferation. In addition, there is evidence of a PGE1 angiogenic capacity in myocardial I/R models (Kawamura et al. 2000; Moeser et al. 2006; Brasileiro et al. 2013). Misoprostol (MSP), a synthetic analog of PGE1, has been used in hepatic I/R models, demonstrating its significant role as a clearing oxidative stress mediator that protects the membrane of endothelial cells. It has also shown antiapoptotic effects in the hepatic sinusoid epithelium generated in the hypoxia and reoxygenation process (Salam et al. 2009; Yang et al. 2002; Lim et al. 1994, 1992; Hardy et al. 1995).

The objective of this study was to evaluate the effect of MSP in the regulation of inflammatory and oxidative stress mediators in I/R-induced renal injury.

2. Investigations and results

Wistar rats were divided into 3 groups. Sham and I/R were given 1 mL/day of physiological solution; MSP+I/R had intragastric administration of MSP (300 μg/kg) for 3 days. In I/R and MSP+IR, the renal hilum was clamped for 45 min, followed by 15 h of reperfusion. Renal function tests, pro-inflammatory cytokines, mediators of oxidative stress, and histological analyses were evaluated.

Table: Renal function markers

	Sham	I/R	MSP+I/R
Creatinine (mg/dL)	0.52±0.07	2.74±0.30 *	1.38±0.45 * *
BUN (mg/dL)	12±02	82±14	78±22

BUN, blood urea nitrogen; I/R, ischemia/reperfusion; MSP, misoprostol; * Sham vs. I/R, p<0.001; ** I/R vs. MSP, p<0.001.

2.1. *Serum creatinine and blood urea nitrogen levels*

Serum creatinine levels after 15 h of reperfusion were significantly higher in I/R vs. Sham ($p<0.001$). MSP+I/R significantly decreased the creatinine concentration compared with I/R ($p<0.001$). However, no significant difference was observed in blood urea nitrogen (BUN) levels between I/R and MSP+I/R (p=0.873) (Table).

Interleukin 6 (IL-6), interleukin 1-β (IL-1b), and tumor necrosis factor alpha (TNF-α) differed significantly between Sham vs. I/R (IL-6: 0.13±0.02 ng/mL vs. 0.39±0.06 ng/mL, p<0.001; IL-1β: 0.9±0.13 ng/mL vs. 1.33±0.09 ng/mL, p<0.001; TNF-α: 0.4±0.07 ng/mL vs. 0.69±0.15 ng/mL, p<0.001) and between I/R vs. MSP+I/R (IL-6: 0.39±0.06 ng/mL vs. 0.20±0.04 ng/mL, p=0.0079; IL-1β: 1.33±0.09 ng/mL vs. 0.73±0.1 ng/mL, p=0.0079; TNF-α: 0.69±0.15 ng/mL vs. 0.43±0.08 ng/mL, p<0.001). No significant difference was found between Sham vs. MSP+I/R (Fig. 1).

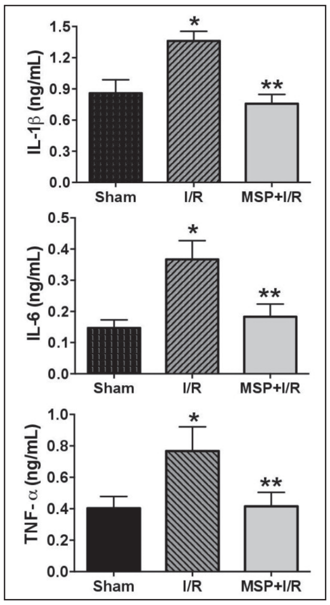

Fig. 1: Pro-inflammatory cytokines

2.2. *Mediators of oxidative stress*

Serological quantification of MDA differed significantly between Sham vs. I/R (235.9±67.1 µM vs. 1341.1±191.1 µM); I/R vs. MSP+I/R (1341.1±191.1 µM vs. 809.1±77.6 µM) and Sham vs. MSP+I/R (235.9±67 µM vs. 809.1±77.6 µM) (all p<0.001). SOD levels differed significantly between Sham vs. I/R (1140.1±19.7 U/mL vs. 926.6±19.7 U/mL, p<0.001); Sham vs. MSP+I/R (1140.1±19.7 U/mL vs. 1360.2±19.7 U/mL, p<0.001) and I/R vs. MSP+I/R (926.6±19.7 U/mL, vs. 1360.2±19.7 U/mL, p<0.001) (Fig. 2).

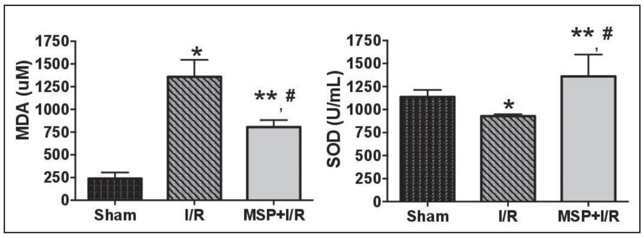

Fig. 2: Oxidative stress markers

2.3. *Histopathological changes*

Histological analysis of the kidneys in the Sham group (Fig. 3A) showed a normal renal parenchyma. After ischemic kidney injury in the I/R group, changes in architecture were observed with the presence of necrosis in the tubular epithelium, mainly in the renal medulla that extended to the renal cortex. There were also abundant cylinders, mainly in the medulla; the glomeruli and blood vessels were preserved (Fig. 3B). Minor, thin, irregular areas of necrosis in the renal medulla without cortical necrosis were observed in the MSP+I/R kidneys; the other structural components were preserved (Fig. 3C).

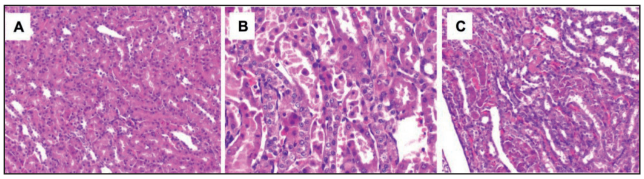

Fig. 3: Hematoxylin and eosin-stained rat kidney sections

3. Discussion

MSP, a synthetic analog of PGE1, has shown a beneficial effect for modulating the inflammatory response in several models. Haynes et al. (1992) showed that MSP inhibits the production of TNF-α and IL-1β and increases the production of IL-6 in isolated rat and mouse macrophages stimulated with lipopolysaccharide (LPS). Martin et al. (2017) showed that pre- and posttreatment with 100 mM of MSP significantly inhibited production of mRNA and pro-inflammatory cytokines TNF-α and IL-6 in equine leukocytes stimulated with (LPS) in an *in vitro* inflammation model, showing its therapeutic effect. In a mouse nephritis model MRL-lpr/lpr, the effect of MSP (1 mg/kg s.c. twice daily for two days) showed a short-term change in production of inflammatory mediators involved in its pathogenesis, suggesting that treatment with MSP transiently reduced IL-1 mRNA levels (Fan et al. 1995).

In an I/R-induced liver injury model, it was reported that MSP attenuates the inflammatory response in the initial phase of reperfusion, inhibiting the release of inflammatory mediators such as TNF-α and IL-1β in Kupffer cells, and activation of neutrophils and endothelial cells (Colleti et al. 1990; Suzuki et al. 1994). To our knowledge, the effect of MSP on regulation of pro-inflammatory cytokines in renal I/R has not been previously described. The current study shows that serum levels of TNF-α, IL-1β, and IL-6 are attenuated by administration of MSP (300 µg/kg for 3 days and 1 h before ischemia) in renal I/R, with values not statistically different from the Sham group.

Based on the biochemical response to renal I/R, the ability of MSP to protect the kidney against ischemic and toxic renal injury has also been evaluated. MSP-treated rats (333 µg/kg *via* orogastric tube 30 min before I/R [40 min/24 h]) had glomerular filtration rates (inulin clearance) almost threefold greater than the I/R group, although renal blood flow and renal vascular resistance did not differ significantly. The improved tubular function was reflected in a lower fractional excretion of sodium and higher urine-to-plasma creatinine ratio. MSP also provided similar protection in a model of toxic renal injury produced by mercuric chloride (1.5 mg/kg and evaluated at 24 h post-I/R) (Paller et al. 1992). Our results were similar regarding renal function, since serum creatinine levels decreased considerably with administration of MSP after ischemic injury.

Other pathways involved in I/R injury include increased oxidative stress due to an alteration in the apoptotic signaling pathways and DNA damage by increasing ROS. In mouse liver cell culture, the antioxidant effects of MSP prevented tissue injury by reducing free oxygen radicals after administration of carbon tetrachloride (Slam et al. 2009) and acetaminophen (Lim et al. 1994). In an *in vitro* model with equine neutrophils challenged with LPS, MSP treatment led to lower ROS production. In another hepatic I/R model, MSP decreased transaminase levels (Lim et al. 1992; Hafez et al. 2007) and prevented microvascular changes (Lim et al. 1994), modifying the effects of reactive oxygen metabolites. In a Wistar rat model, MSP was evaluated (50 µg/kg/d for 3 days) in I/R (45 min/60 min)-induced intestinal injury. In ileum tissue, MDA was found to be higher in I/R compared with sham, but did not differ from I/R+MSP; meanwhile SOD did not differ between sham and I/R+MSP, but was significantly higher in I/R (Topcu et al. 2007). Our study showed that MSP treatment increases antioxidant activity, with lower MDA levels and increased SOD activity in renal tissue.

Renal ischemia induced for 45 min followed by 15 h of reperfusion in our model resulted in massive renal tissue injury, indicated by tubular epithelium necrosis shown in marrow and cortex, which was significantly reduced with MSP administration. Previous studies have suggested that MSP prevents injury by common protection mechanics. Paller et al. (1992) reported that MSP-treated cells had microvilli preservation and intact apical membranes, unlike (*in vitro*) renal control cells subjected to hypoxia and reoxygenation, which showed extensive structural changes. Disruption of the apical membrane, an irreversible change, was not seen in any MSP-treated cells. In another hepatic I/R model (beagle dogs), MSP administration by continuous i.v. infusion for 30 min before ischemia onset (15 µg/kg) and from 15 min before reperfusion for 3 h (35 µg/kg) improved hepatic sinusoidal congestion, derangement, ballooning, hepatocyte necrosis bridging, and endothelial cell detachment observed in the I/R group (Totsuka et al. 1998). However, in another study, the hepatoprotective effects of MSP against I/R injury, studied using partial liver ischemia in a rat model, indicated that MSP offers only partial protection, with some signs of tissue necrosis (Lim et al. 1992). Our investigation confirms the nephroprotective effect of MSP against ischemic injury in rats. In conclusion, MSP attenuates renal I/R injury through the modulation of pro-inflammatory cytokines and oxidative stress mediators, thereby protecting renal tissue from the ischemic lesion.

4. Experimental

4.1. Animals

Management of the animals was carried out with approval by our institution's ethics committee (H17-00002) and following the guidelines for the care of laboratory animals included in the Official Mexican Standard NOM-062-ZOO-1999. The animals were kept at a stable ambient temperature, with circadian cycles (12 h light: 12 h dark) and were provided food and water *ad libitum*.

4.2. Study design

Male Wistar rats (n=18) 200–300 g were used. Three comparative groups (each n=6) were formed. Control group (Sham): Physiological solution (1 mL/day) administered intragastrically for 3 days at 24 h intervals and 1 h before the laparotomy procedure. Ischemia/reperfusion group (I/R): Physiological solution (1 mL/day) administered intragastrically for 3 days at 24 h intervals and 1 h before the renal I/R procedure. I/R+MSP group: MSP (Cytotec, Pfizer Inc., Mexico) (300 µg/kg) administered intragastrically for 3 days at 24 h intervals and 1 h before the renal I/R procedure.

4.3. Kidney I/R model

Prior to the procedure, the animals were subjected to 8 h of fasting, then anesthetized with intraperitoneal xylazine (10 mg/kg) (Sedaject, Vedilab SA de CV Reg. SAGARPA Q-008 8-122) and ketamine (100 mg/kg) (Anesket, PiSA Agropecuaria, SA de CV Reg. SAGARPA Q7833-028).

In all groups, animals were subjected to a longitudinal midline incision laparotomy, realizing minimal dissection of muscular and s.c. tissue, after which both renal hilum were carefully exposed. To induce renal ischemia in I/R and MSP+I/R, microvascular clamps were placed bilaterally (Aesculap, San Francisco, CA, USA), maintaining vascular occlusion for 45 min, ensuring a macroscopic change of the renal parenchyma. Next, all clamps were detached, starting a 15 h reperfusion.

4.4. Sample collection

The animals were sacrificed and blood samples and renal tissues were collected. Blood samples were obtained by catheterization of the inferior vena cava and then centrifuged at 3,500 rpm for 15 min to collect serum. The tissue samples were divided proportionally for histopathological analysis (fixed in 10% formaldehyde with phosphate buffer) and oxidative stress marker levels (frozen at –70 °C).

4.5. Biochemical analysis

Conventional test kits were used to evaluate creatinine and BUN levels (ILab Aries, Instrumentation Laboratory, Italy).

4.6. Inflammatory mediators

Serum levels of TNF-α, IL-β, and IL-6 were determined by an enzyme-linked immunosorbent assay (ELISA) using test kits (Peprotech, Mexico).

4.7. Oxidative stress markers

MDA determination was carried out with the trichloroacetic acid method, which is based on the reaction of MDA with thiobarbituric acid in an acidic and hot medium (Yagy 1995), using a commercial MDA assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). SOD levels were determined by reducing tetrazolium salt to a formazan in the presence of xanthine oxidase, xanthine, and oxygen. The presence of SOD slows the tetrazolium reduction and was measured via spectrophotometry (Sandström et al. 1994) using a commercial test kit for SOD (Cayman Chemical Company).

4.8. Histopathological evaluation

Tissue samples conserved in 10% formaldehyde were processed and fixed in paraffin for histopathological review. Conventional staining with hematoxylin and eosin (H&E) was performed and examined with an optical microscope by a single pathologist.

4.9. Statistical analysis

Statistical analyses were run using GraphPad Prism 7.0 software. The Kruskal–Wallis test was used for nonparametric data and the Mann–Whitney *U* test for group comparison. Values are expressed as the mean ± standard deviation (SD) and p-values <0.05 were considered statistically significant.

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Conflicts of interest: None declared.

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