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Recovery From Glycerol-Induced Acute Kidney Injury Is Accelerated By Suramin.

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Running title page

SURAMIN AND GLYCEROL-INDUCED AKI

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List of nonstandard abbreviations:

1) AKI, acute kidney injury; 2) GAPDH, glyceraldehyde 3-phosphate dehydrogenase; 3) HO-1, Hemeoxygenase-1; 4) IL-6/1β, Interleukins–6 and 1 beta; 5) ICAM-1, Intracellular adhesion molecule-1; 6) MCP-1, Monocyte chemoattractant protein-1; 7) NGAL, Neutrophil gelatinase-associated lipocalin 2; 8) PCNA, Proliferating cell nuclear antigen; 9) Phospho-p65, phosphorylated-p65; 10) TGF- β_1, Transforming growth factor-beta 1; 11) TNFα, Tumor necrosis factor alpha.

ABSTRACT

Acute kidney injury (AKI) is a common and potentially life-threatening complication following ischemia/reperfusion (IR) and exposure to nephrotoxic agents. In this study, we examined the efficacy and mechanism(s) of suramin in promoting recovery from glycerol-induced AKI, a model of rhabdomyolysis-induced AKI. Following intramuscular glycerol injection (10 ml 50% glycerol/kg) into male Sprague Dawley rats, serum creatinine maximally increased at 24-72 h and then decreased at 120 h. Creatinine clearance (CrCl) decreased 75% at 24-72 h and increased at 120 h. Suramin (1 mg/kg, iv) administered 24 h after glycerol, accelerated recovery of renal function as evidenced by increased CrCl, decreased renal kidney injury molecule-1 and improved histopathology 72 h after glycerol injection. Suramin treatment decreased interleukin-1 beta (IL-1β) mRNA, transforming growth factor-β_1 (TGF-β_1), phospho-65 of nuclear factor κB (NFκB) and cleaved caspase-3 at 48 h when compared to glycerol alone. Suramin treatment also decreased glycerol-induced activation of intracellular adhesion molecule-1 (ICAM-1) and leukocyte infiltration at 72 h. Urinary/renal neutrophil gelatinase-associated lipocalin 2 (NGAL) levels, hemeoxygenase-1 expression and renal cell proliferation were increased by suramin compared to glycerol alone at 72 h. Mechanistically, suramin decreases early glycerol-induced pro-inflammatory (IL-1β and NFκB) and growth inhibitory (TGF-β_1) mediators resulting in prevention of late downstream inflammatory effects (ICAM-1 and leukocyte infiltration) and increasing compensatory nephrogenic repair. These results support the hypothesis that delayed administration of suramin is effective in abrogating apoptosis, attenuating inflammation and enhancing nephrogenic repair following glycerol-induced AKI.

Introduction

Acute kidney injury (AKI) is a critical clinical problem with a high mortality rate and can occur following an acute injury or as a silent event, being identified only after its occurrence by the onset of progressive azotemia (Schrier et al., 2004). Rhabdomyolysis-induced AKI, also termed “crush” kidney injury, develops following skeletal muscle trauma related to physical, thermal, ischemic, infective, metabolic or toxic causes, releasing toxic doses of myoglobin and other intracellular proteins into the circulation (Vanholder et al., 2000; Huerta-Alardin et al., 2005; Bagley et al., 2007). Approximately 10 to 50% of patients suffering from rhabdomyolysis develop some degree of AKI (Ward, 1988) and although interventions have improved, the mortality rate may still be as high as 8% (Polderman, 2004; Huerta-Alardin et al., 2005; Bagley et al., 2007). Animal models of glycerol-induced AKI are currently being used to understand the clinical syndrome and study the mechanisms of AKI in general (Nath et al., 1992).

While the pathogenesis of glycerol-induced AKI is complex and incompletely understood (Vanholder et al., 2000), apoptosis (Homsi et al., 2010; Kim et al., 2010; Wang et al., 2011), inflammation (Homsi et al., 2006) and oxidative stress (Rosenberger et al., 2008; Kim et al., 2010; Wei et al., 2011) have been implicated. Recent studies have demonstrated that timely prophylactic and/or early therapeutic interventions (either pre- or co-administration) ameliorated glycerol-induced AKI (Homsi et al., 2010; Kim et al., 2010; Wang et al., 2011; Wei et al., 2011). However, no reports have documented the efficacy of a delayed intervention of a therapeutic agent when the renal dysfunction is already well established.

Recently, it was reported that delayed administration of suramin, a polysulfonated naphthylurea used in humans to treat trypanosomiasis, accelerates recovery from renal dysfunction induced by ischemia reperfusion (IR) injury, renal fibrosis and obstructive nephropathies in mice (Zhuang and Schnellmann, 2005; Zhuang et al., 2009; Liu et al., 2011a; Liu et al., 2011b). The mechanisms by which suramin accelerates recovery from renal dysfunction in these models remain incompletely understood, but may be associated with inhibition of apoptosis, suppression of inflammatory cytokine production and promoting epithelial cell proliferation. However, it is unknown whether delayed administration of suramin is beneficial in other forms of AKI (i.e. glycerol-induced AKI).

In this study, we examined the effect of delayed administration of suramin on renal function and pathology in a rat model of glycerol-induced AKI. We examined the more clinically relevant question of whether suramin promotes recovery from glycerol-induced renal dysfunction by administering suramin 24 h after AKI, when serum creatinine levels were maximal. We also uncovered some novel mechanisms underlying glycerol-induced AKI and the effects of suramin on predictive biomarkers of AKI, signaling involved in leukocyte infiltration and inflammation, oxidative stress and tubular cell proliferation.

Methods

Animals and treatment

Male Sprague–Dawley (SD) rats (Harlan Laboratories, Madison, WI), 8 weeks of age (180-200 g), were housed in temperature-controlled conditions under a light/dark photocycle with food and water supplied *ad libitum*. Rats were dehydrated for 16 h before glycerol injection. Rats were divided randomly into three groups. The first group (Untreated, n = 5) was not injected with any treatment; the second and third groups of rats (n = 20) were injected intramuscularly (IM) with 50% glycerol (10 ml/kg) into hind limbs. The first and second groups received sterile water and the third group received suramin intravenously (IV) (1 mg/kg, dissolved in sterile water) 24 h after initial glycerol injection. Blood, urine and kidney samples were collected at 24, 48, 72 and 120 h. All animal and treatment protocols were in compliance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and were approved by our Institutional Animal Care and Use Committee (IACUC).

Chemicals

Unless stated otherwise, all chemicals and biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Goat polyclonal anti-kidney injury molecule-1 (KIM-1) antibody was obtained from R&D systems (Minneapolis, MN). Rabbit polyclonal anti-NGAL and mouse monoclonal anti-transforming growth factor-β_1 (TGF- β_1) antibodies were purchased from Abcam Inc. (Cambridge, MA), rabbit polyclonal anti-caspase-3 was obtained from Enzo Life Sciences (Farmingdale, NY), rabbit polyclonal anti-intracellular adhesion molecule-1 (ICAM-1) and rabbit polyclonal anti-proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit monoclonal anti-

phospho-p65 and rabbit monoclonal anti-hemeoxygenase-1 (HO-1) were obtained from Cell Signaling Technology (Beverly, MA), and the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Fitzgerald International Inc. (Acton, MA). Anti-goat secondary antibody conjugated with horseradish peroxidase was purchased from Millipore (Billerica, MA), and anti-rabbit and anti-mouse secondary antibodies conjugated with horseradish peroxidase were obtained from Pierce (Rockford, IL).

Assessment of renal function

Rats were placed in metabolic cages (Tecniplast, Philadelphia, PA) for 24 h urine collections. Renal function was monitored by measuring 24 h urine volume, serum and urine creatinine using a creatinine assay kit (BioAssay Systems, Hayward, CA) as per manufacturer's instructions and creatinine clearance (CrCl) was calculated. Urinary neutrophil gelatinase-associated lipocalin (NGAL) was measured using an ELISA (ALPCO Immunoassays, Salem, NH) as per manufacturer's protocol.

Histology and immunohistochemistry

Renal tissues were fixed in 4.5% buffered formalin, dehydrated, and embedded in paraffin. For general histology, sections were stained with hematoxylin/eosin. For immunohistochemistry of KIM-1, NGAL, ICAM-1 and PCNA, manufacturer's protocols were followed.

Assessment of renal inflammation

Renal inflammation was assessed by measuring leukocyte (neutrophils, monocytes) infiltration using the naphthol AS-D chloroacetate esterase kit (Sigma Chemical Co., St. Louis, MO) and immunohistochemistry carried out as per manufacturer's protocol. To quantify leukocyte infiltration, a total of 25 fields (original magnification ×20) in the outer

stripe of outer medulla (OSOM) were examined and expressed as the total number of leukocytes in all the fields.

Quantitative PCR

Rat kidney cortex samples were homogenized with TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. The mRNA was subjected to reverse transcription to cDNA in the presence of oligo dT primers using RevertAid First Strand cDNA synthesis kit from Fermentas Life Sciences (Glen Burnie, MD). PCR was performed using commercially available primers (Integrated DNA Technologies, Coraville, IA) for TNFα, IL-6, IL-1β, MCP-1 and tubulin. Tubulin was used to normalize mRNA expression.

Immunoblot analysis

Rat kidney cortex tissue was homogenized in 5 volumes of protein lysis buffer (1% Triton X 100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM EGTA; 2 mM sodium orthovanadate; 0.2 mM phenylmethylsulfonylfluoride; 1 mM HEPES pH 7.6; 1 μg/ml leupeptin; and 1 μg/ml aprotinin) using a polytron homogenizer. The homogenate was stored on ice for 10 min and then centrifuged at 7,500 *g* for 5 min at 4^{o}C. The supernatant was collected and protein determined using a Bicinchoninic acid kit (Sigma Chemical Co, St. Louis, MO) with bovine serum albumin as the standard. Proteins (50-75 μg) were separated on 4-20% gradient sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked either in 5% dried milk or BSA in TBST (0.1% Tween 20 in 1x TBS) and incubated with 1:1000 dilutions of anti-KIM-1, anti-NGAL, anti- TGF- β_1, anti-caspase-3, anti-phospho-p65, anti-HO-1, and anti-GAPDH; and 1:100 dilutions of anti-ICAM-1 and anti-PCNA overnight at 4^{o}C. After

incubation for 2 h at room temperature with secondary antibodies (1:2000) conjugated with horseradish peroxidase, membranes were detected by chemiluminiscence.

Data and Statistical analysis

Data are expressed as means ± SEM (n = 4-5) for all the experiments. Multiple comparisons of normally distributed data were analyzed by one-way ANOVA, as appropriate, and group means were compared using Student-Newman-Keuls post-hoc test. Single comparisons were analyzed by Student's *t*-test where appropriate. The criterion for statistical differences was $p \leq 0.05$ for all comparisons.

Results

Suramin improves glycerol-induced renal dysfunction and damage. Serum creatinine was maximal and sustained from 24-72 h, and decreased to slightly higher than control levels at 120 h following glycerol treatment (Fig. 1 A). Urine creatinine decreased similarly after glycerol in the presence and absence of suramin 24-120 h when compared to untreated controls (Fig. 1 B). Additionally, urine volume increased after glycerol in the presence and absence of suramin 48-120 h when compared to untreated controls (Fig. 1 C). CrCl decreased 75% and remained inhibited at 24-72 h and returned to control levels at 120 h after glycerol alone (Fig. 1 D). However, while CrCl was similar to glycerol alone at 48 h, suramin treatment increased CrCl to control levels at 72 and 120 h (Fig. 1 D).

Associated with the renal dysfunction, glycerol-treated rats exhibited extensive proximal tubular necrosis throughout the corticomedullary region characterized by eosinophilic tubules with remnants of karyolytic nuclei (Fig. 2 B) when compared to the renal architecture in control animals (Fig. 2 A). There was minimal to no histological appearance of repair defined by a thin layer of cuboidal re-epithelialization in the tubular lamina at 72 h. Administration of suramin 24 h after glycerol treatment, resulted in decreased pathology and clear evidence of renal tubular repair characterized by marked renal tubule basophilia and squamous to low cuboidal re-epithelialization inside residual tubular basal lamina (Fig. 2 C).

Suramin intervention decreases KIM-1 expression following glycerol-induced AKI. KIM-1 protein expression in the kidney was elevated 24 h after glycerol alone and was sustained until 72 h (Fig. 3 A & B). With suramin treatment, renal KIM-1 levels were lower at 72 h after glycerol administration. KIM-1 expression was not detected in untreated rat kidneys by immunohistochemical staining. In glycerol-treated rat kidneys, KIM-1 staining increased and

was mainly localized to the apical membranes of proximal tubular epithelial cells (arrows) along with some damaged tubules at 72 h (Fig. 3 D). In contrast, glycerol + suramin rats exhibited less KIM-1 staining distributed in tubular epithelial cells and there were less KIM-1 positive tubules when compared to glycerol-treated rat kidneys at 72 h (Fig. 3 E).

Suramin increases urinary and renal NGAL levels following glycerol-induced AKI. Urinary NGAL in glycerol-treated rats was elevated when compared to control rats with maximal levels at 48 h after glycerol and NGAL remained elevated through 120 h (Fig. 4 A). Renal protein and tissue NGAL did not increase until 48 h following glycerol treatment (Fig. 4 B-E). After suramin treatment, urinary NGAL was higher at 48 h and 72 h (Fig. 4 A), and renal protein and tissue NGAL staining was higher at 48 h compared to glycerol alone-treated rats (Fig. 4 B-F).

Suramin decreases apoptosis following glycerol-induced AKI. Caspase-3-mediated apoptosis has been implicated in tubular damage after glycerol-induced AKI (Padanilam, 2003; Homsi et al., 2010; Wang et al., 2011). Suramin decreased renal cleaved caspase-3 at 48 h after glycerol when compared with glycerol alone at the same time point (Fig. 5 A & B). However, there was no difference in cleaved caspase-3 among control and glycerol-treated rats ± suramin at 72 h (densitometric units of renal cleaved caspase-3 normalized to GAPDH for Control: 0.45 ± 0.01; Glycerol alone: 0.94 ± 0.16; and Glycerol + Suramin: 0.67 ± 0.14 at 72 h).

Suramin decreases leukocyte infiltration and inflammation after glycerol-induced AKI. Leukocyte-mediated infiltration and inflammation has been suggested to be an important event in AKI (Bonventre and Zuk, 2004; Homsi et al., 2006; de Jesus Soares et al., 2007). We analyzed leukocyte infiltration following glycerol-induced AKI in the corticomedullary region

of kidneys of rats treated with or without suramin by staining neutrophils and monocytes with naphthol AS-D chloroacetate esterase as described previously (Zhuang et al., 2009). Increased leukocytes were observed at 72 h after glycerol alone treatment (Fig. 6 B & D) and were decreased with suramin intervention at the same time point (Fig. 6 C & D).

Suramin attenuates ICAM-1 expression following glycerol-induced AKI. Activation of renal endothelial cell ICAM-1 expression by leukocytes and their contribution in the pathophysiology of AKI in general and glycerol-induced tubular inflammation and necrosis, have been previously documented (Liu et al., 2002; Bonventre, 2010). We did not find any difference in ICAM-1 expression among untreated and glycerol-treated rats ± suramin at 0-48 h (densitometric units of renal ICAM-1 normalized to GAPDH for Control: 0.6 ± 0.2; 24 h glycerol alone: 0.8 ± 0.3; 48 h Glycerol alone: 0.6 ± 0.2; 48 h Glycerol + Suramin: 0.4 ± 0.1). However, suramin inhibited ICAM-1 expression at 72 h after glycerol when compared to glycerol alone (Fig. 7 A & B). ICAM-1 localization at the basement membranes of proximal convoluted tubules was blocked by suramin treatment when compared to glycerol alone (Fig. 7 D & E).

Suramin decreases pro-inflammatory signaling following glycerol-induced AKI. The role of innate immunity and the pro-inflammatory mediators in progression of renal inflammation in AKI has been established (Homsi et al., 2009; Goncalves et al., 2010). There were no differences in mRNA levels of renal TNFα and MCP-1 after glycerol with or without suramin over the time course (Fig. 8 A & D). However, renal IL-6 mRNA at 48 h in glycerol-treated rats with or without suramin was higher when compared to untreated rats (Fig. 8 B). Interestingly, renal IL-1β increased numerically, but not statistically, 24 and 48 h after

glycerol administration and suramin decreased IL-1β to below control levels at 48 h (Fig. 8 C).

Suramin decreases NFκB activation following glycerol-induced AKI. Phosphorylation of the p65 subunit of NFκB leads to nuclear translocation of NFκB and transcription of several genes involved in renal inflammation (Hu et al., 2011). ICAM-1 is one of those genes which is positively regulated by NFκB (Zheng et al., 2006; Yang et al., 2010; Chen et al., 2011; Li et al., 2011). Phosphorylated-p65 was increased at 24 and 48 h in glycerol-treated rats when compared to untreated rats (Figs. 9 A & B) and suramin treatment after glycerol decreased the phosphorylation of p65 at 48 h when compared to glycerol alone (Figs. 9 A & B).

Suramin increases HO-1 and decreases TGF-β_1 following glycerol-induced AKI. HO-1 was shown to be indispensable in protecting against glycerol-induced AKI (Ishizuka et al., 1997; Nath et al., 2000; Rosenberger et al., 2008; Wei et al., 2011). Renal HO-1 protein was decreased after glycerol from 24 h through 72 h. However, with suramin treatment, HO-1 increased at 72 h when compared to glycerol alone (Figs. 10 A & B). Suramin was previously reported to inhibit fibrotic and growth inhibitory actions of TGF-β_1 (Liu et al., 2011a; Liu et al., 2011b; Liu and Zhuang, 2011). In this study we found that suramin decreased TGF-β_1 protein at 48 and 72 h after glycerol when compared to the glycerol alone (Figs. 10 C & D).

Suramin increases tubular cell proliferation following glycerol-induced AKI. Kidneys from rats treated with glycerol and glycerol + suramin exhibited similar increases in PCNA protein expression at 24 and 48 h. (Figs. 11 A & B). However, at 72 h post glycerol treatment, PCNA protein expression remained elevated in the glycerol + suramin group while it decreased in the glycerol group (Figs. 11 A & B). Immunohistochemically, there was an increase in number of PCNA-positive proximal tubular epithelial cells and this increase was

sustained until 72 h with suramin treatment when compared to glycerol alone-treated rats (Figs. 11 C, D, & E).

Discussion

Previous studies have determined that therapeutic interventional strategies before, at the time of I/R, drug, or toxicant exposure or within a few hours after exposure attenuate AKI (Kelly et al., 1994; Chiao et al., 1997; Bates and Lin, 2005; Johnson et al., 2006). Recently, it was demonstrated that delayed administration of suramin after renal dysfunction was established (24 h) accelerated recovery of renal function (Zhuang et al., 2009; Liu et al., 2011a; Liu et al., 2011b). This is of therapeutic importance because most cases of AKI in the clinical setting are not identified until sometime after the insult has already occurred. Here, we demonstrate the beneficial effects of delayed administration (24 h) of suramin and identified novel protective mechanisms in another clinically relevant model of AKI (rhabdomyolysis-induced AKI). Furthermore, we demonstrate that suramin accelerates recovery of renal damage and function by restoring renal architecture following tubular necrosis. Suramin exerts this beneficial effect through inhibition of apoptosis, decreased activation of endothelial cells, decreased inflammatory signaling molecules and infiltration of inflammatory cells, decreased oxidative stress and increased tubular cell proliferation. These results are consistent with previous studies of glycerol-induced AKI in which oxidative stress, apoptosis and inflammation are key mediators of renal dysfunction (Homsi et al., 2006; Rosenberger et al., 2008; Homsi et al., 2010; Kim et al., 2010; Wang et al., 2011; Wei et al., 2011).

A pro-inflammatory response and leukocyte infiltration is an important mechanism in the initiation and maintenance of glycerol-induced AKI (Bonventre and Zuk, 2004; Homsi et al., 2006; de Jesus Soares et al., 2007). We did not observe a difference in the mRNA levels of TNFα and MCP-1 after glycerol, which was shown previously in glycerol-induced AKI (Homsi et al., 2009). However, IL-1β mRNA increased 24 h and IL-6 mRNA increased 48 h

after glycerol treatment and suramin treatment blocked the increase in IL-1β. Suramin did not statistically block the increase in IL-6. While IL-6 has been reported to play a role in AKI by increasing a cell-mediated immune response that promotes renal injury and a protective response to tubular cells that maintains renal function (Nechemia-Arbely et al., 2008), the exact role of IL-6 in these experiments remains unclear.

IL-1β processing and activation has been shown to increase nuclear translocation of NF-κB (phosphorylation of p65 subunit) in endothelial cells resulting in transcriptional activation of adhesion molecules like ICAM-1 (Yang et al., 2010). Circulating leukocytes use endothelial cell transmembrane ICAM-1 as an anchor to transmigrate and cause inflammation (Lai et al., 2003; Nayak, 2005; Iwai et al., 2006; Yang et al., 2010). We found that IL-1β mRNA levels increased at 24-48 h and phospho-p65 (activated NF-κB) increased at 24-48 h after glycerol alone, that suramin treatment at 24 h after glycerol decreased IL-1β mRNA and phospho-p65 at 48 h, and that ICAM-1 and leukocyte infiltration were decreased at 72 h. These sequential events may result from the suppressive effect of suramin on IL-1β mRNA or a direct inhibitory effect on NF-κB activation as previously reported (Goto et al., 2006). Thus, the sequence of events aligns and we suggest that activation of upstream and early events like IL-1β (24-48 h) and NF-kB (24-48 h) may be responsible for the up regulation of ICAM-1 (72 h) after glycerol alone. The finding of ICAM-1 blockade leading to decreased leukocyte adhesion and renal inflammation is consistent with a previous report that showed an antibody directed against ICAM-1 protected the kidney against I/R injury in rats (Kelly et al., 1994). It is also possible that suramin may block toll-like receptor 4, which is known to trigger an inflammatory response by activating IL-1β and increasing nuclear translocation of NF-κB (Goncalves et al., 2010).

HO-1 protects against oxidative stress and inflammation during AKI (Ishizuka et al., 1997; Nath et al., 2000; Rosenberger et al., 2008; Bolisetty et al., 2010; Wei et al., 2011; Zarjou and Agarwal, 2011). We found that HO-1 decreased 24 h following glycerol treatment and remained decreased through 72 h, and that suramin up-regulated renal HO-1 at 72 h after glycerol. One possible explanation for this observation could involve NGAL since NGAL was previously shown to confer a cytoprotective and survival benefit in response to diverse forms of cellular stress by inducing HO-1 (Mori et al., 2005; Schmidt-Ott et al., 2007; Bahmani et al., 2010; Johnson et al., 2010). Consistent with this idea, we found that suramin markedly increased renal NGAL levels 48 h after glycerol, prior to the suramin-induced increase in HO-1 at 72 h.

We also observed that urinary NGAL increased within 24 h after glycerol and increased further at 48 h while renal NGAL did not change at 24 or 48 h. Suramin intervention increased NGAL in urine and renal cortex at 48 h after glycerol. NGAL is currently thought to maintain the balance in iron levels in cells (Johnson et al., 2010). Thus it seems possible that when proximal tubular cells are exposed to iron-mediated oxidative stress, (resulting from glycerol-mediated rhabdomyolysis and release of heme from circulating myoglobin in the tubular lumen) increased NGAL may be produced. It is also documented that NGAL is up-regulated during the time of kidney damage and participates in nephrogenic repair and regeneration (Mishra et al., 2003; Mishra et al., 2004; Mori et al., 2005; Schmidt-Ott et al., 2007; Sola et al., 2011). However, the mechanism(s) by which suramin increased proximal tubular NGAL expression currently remains unknown. One hypothesis is that suramin may increase the expression of receptors for NGAL uptake in the proximal tubules. Megalin/24p3R receptor-mediated proximal tubular uptake of NGAL has been suggested (Schmidt-Ott et al., 2007).

It has been previously documented that an inflammatory reaction following muscle injury, releases TGF-β_1 resulting in fibrosis *via* the activation of fibroblasts and production of extracellular matrix which halters muscle regeneration and recovery. Suramin administration was shown to significantly decrease the stimulating effect of TGF-β_1 on the growth of muscle-derived fibroblasts and accelerated muscle recovery (Chan et al., 2003; Nozaki et al., 2008). In our study, TGF-β_1 increased 48 and 72 h following glycerol treatment and suramin blocked the increase in TGF-β_1. This effect of suramin on TGF-β_1 was associated with decreased apoptosis 48 h post glycerol and increased proximal tubular cellular proliferation. This finding is consistent with our previous data showing that TGF-β_1 induces apoptosis in RPTC and that suramin stimulates proliferation of RPTC in primary cultures by activating Src-PI3/Akt pathway (Zhuang and Schnellmann, 2005).

In addition to being a sensitive biokmarker of kidney injury (Bonventre, 2008), KIM-1 may play a role in repair (Ichimura et al., 1998; Bailly et al., 2002; Huo et al., 2010) following AKI. We observed increased and sustained expression of KIM-1 in glycerol alone-treated group 24-72 h and suramin treatment decreased KIM-1 at 72 h. Thus, it is unlikely that KIM-1 plays a role in regeneration in this model since KIM-1 decreased when suramin-induced stimulation of regeneration remains high.

In summary, we found that suramin exerts positive effects on three different endpoints to promote recovery from glycerol-induced AKI. Suramin inhibited the delayed apoptosis, decreased inflammation and promoted proliferation of the renal tubular epithelial cells. Further exploration of the signaling pathways revealed that suramin decreased pro-inflammatory IL-1β and NF-κB and growth inhibitory TGF-β_1 48 h after glycerol, thereby attenuating subsequent inflammation as evidenced by a decrease in leukocyte infiltration and ICAM-1 expression at 72

h. In addition, suramin increased the anti-inflammatory protein HO-1 and increased cell proliferation at 72 h after glycerol. Therefore, delayed administration of suramin, when serum creatinine levels were maximal, markedly promoted recovery after glycerol-induced AKI. These broad actions of a single, low-dose of suramin might prove to have therapeutic potential against rhabdomyolysis-induced and other forms of AKI in clinical settings.

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DISCLAIMER

The contents of this manuscript do not represent the views of the Department of Veteran Affairs or the United States Government.

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Authorship Contribution

Participated in research design: Rick G. Schnellmann & Midhun C. Korrapati

Conducted experiments: Midhun C. Korrapati & Brooke E. Shaner

Contributed new reagents or analytic tools: None

Performed data analysis: Midhun C. Korrapati, Brooke E. Shaner & Rick G. Schnellmann.

Wrote/contributed to writing of the paper: Midhun C. Korrapati & Rick G. Schnellmann

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Footnotes

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Legends for Figures

Fig. 1. Effect of delayed administration of suramin on renal dysfunction in rats subjected to glycerol-induced AKI.

A. Serum creatinine in groups of male Sprague-Dawley rats (n = 5) which were injected with 10 ml 50% glycerol/kg i.m., with or without 1 mg suramin/kg at 24 h i.v. after glycerol. One more group did not receive any treatment. Data are expressed as mean ± SE (n = 5). ***** Statistically significant from respective 0 h controls. # Significantly different from glycerol alone-treated rats at the corresponding time point ($p \leq 0.05$). **B**. Urine creatinine in groups of male Sprague-Dawley rats (n = 5) which were injected with 10 ml 50% glycerol/kg i.m., with or without 1 mg suramin/kg at 24 h i.v. after glycerol. One more group did not receive any treatment. Data are expressed as mean ± SE (n = 5). ***** Statistically significant from respective 0 h controls. ($p \leq 0.05$). **C**. Urine volume in groups of male Sprague-Dawley rats (n = 5) which were injected with 10 ml 50% glycerol/kg i.m., with or without 1 mg suramin/kg at 24 h i.v. after glycerol. One more group did not receive any treatment. Data are expressed as mean ± SE (n = 5). ***** Statistically significant from respective 0 h controls. ($p \leq 0.05$). **D**. Creatinine clearance in groups of male Sprague-Dawley rats (n = 5) which were injected with 10 ml 50% glycerol/kg i.m., with or without 1 mg suramin/kg at 24 h i.v. after glycerol. One more group did not receive any treatment. Data are expressed as mean ± SE (n = 5). ***** Statistically significant from respective 0 h controls. # Significantly different from glycerol alone-treated rats at the corresponding time point. ($p \leq 0.05$).

Fig. 2. Effect of delayed administration of suramin on renal injury in rats subjected to glycerol-induced AKI.

Representative photomicrographs of H&E-stained kidney sections from rats that were untreated (**A**), treated with glycerol alone, at 72 h (**B**) subjected to suramin-intervention after glycerol, at 72 h (**C**). ***** Denotes necrotic tubules. **#** Denotes tubules that are lined with regenerating cells. All fields were chosen from the outer stripe of the outer medulla (OSOM). Original magnification, 200 X.

Fig. 3. Effect of delayed administration of suramin on kidney injury molecule-1 (KIM-1) in rats subjected to glycerol-induced AKI. **A:** Representative Western blot showing KIM-1 protein expression in kidneys of untreated and glycerol-treated rats (n = 4) with or without suramin over a time course. **B:** Densitometric analysis of renal KIM-1 protein expression in untreated, glycerol and suramin intervention after glycerol-treated groups over the time course. Data were normalized by GAPDH which served as internal control. Data are expressed as mean ± SE (n = 4). ***** Statistically significant from respective 0 h controls. # Statistically significant from glycerol alone-treated rats at the corresponding time point ($p \leq$ 0.05). **U**: Untreated; **G**: Glycerol alone-treated group; **GS**: Glycerol + Suramin-treated group. Representative photomicrographs of KIM-1-stained kidney sections from rats that were untreated (**C**); treated with glycerol alone, at 72 h (**D**); subjected to suramin-intervention after glycerol, at 72 h (**E**). KIM-1 positive staining was observed on the proximal tubular epithelial cells and damaged tubules (arrows). All fields were chosen from the outer stripe of the outer medulla (OSOM). Original magnification, 200 X.

Fig. 4. Effect of delayed administration of suramin on neutrophil gelatinase-associated lipocalin (NGAL) in rats subjected to glycerol-induced AKI. **A:** ELISA of urinary NGAL

levels of untreated and glycerol-treated rats (n = 5) with or without suramin over a time course. **B:** Representative Western blot showing NGAL protein expression in kidneys of untreated and glycerol-treated rats (n = 4) with or without suramin over a time course. **C:** Densitometric analysis of renal NGAL protein expression in untreated, glycerol and suramin intervention after glycerol-treated groups over the time course. Data were normalized by GAPDH which served as internal control. Data are expressed as mean ± SE (n = 4). ***** Statistically significant from respective 0 h controls. # Statistically significant from glycerol alone-treated rats at the corresponding time point ($p \leq 0.05$).

Representative photomicrographs of NGAL-stained kidney sections from rats that were untreated (**D**); treated with glycerol alone, at 48 h (**E**); subjected to suramin-intervention after glycerol, at 48 h (**F**). NGAL positive staining was observed on the proximal tubular epithelial cells (arrows). All fields were chosen from the outer stripe of the outer medulla (OSOM). Original magnification, 200 X.

Fig. 5. Effect of delayed administration of suramin on renal cleaved caspase-3 in rats subjected to glycerol-induced AKI. **A:** Representative Western blot showing cleaved-caspase-3 protein expression in kidneys of untreated and glycerol-treated rats (n = 4) with or without suramin over a time course. **B:** Densitometric analysis of renal cleaved caspase-3 protein expression in untreated, glycerol and suramin intervention after glycerol-treated groups over the time course. Data were normalized by GAPDH which served as internal control. Data are expressed as mean ± SE (n = 4). ***** Statistically significant from respective 0 h controls. # Statistically significant from glycerol and suramin-treated rats at the corresponding time point ($p \leq 0.05$).

Fig. 6. Effect of delayed administration of suramin on renal leukocyte infiltration in rats subjected to glycerol-induced AKI. Representative photomicrographs of neutrophil and monocyte staining assessed by formation of stable pinkish-red colored (arrows) complex of free naphthol and diazonium salts following incubation of kidney sections from rats that were untreated (**A**); treated with glycerol alone, at 72 h (**B**); subjected to suramin-intervention after glycerol, at 72 h (**C**) in naphthol AS-D chloroacetate reagent. All fields were chosen from the outer stripe of the outer medulla (OSOM). Original magnification, 200 X. **D**. Quantitative analysis of renal leukocyte infiltration assessed by number of pink colored dots in a total of 25 fields in the OSOM region of kidney sections. Data are expressed as mean ± SE (n = 4). ***** Statistically significant from respective 0 h controls. # Statistically significant from glycerol alone-treated rats at the corresponding time point ($p \leq 0.05$).

Fig. 7. Effect of delayed administration of suramin on renal intracellular adhesion molecule-1 (ICAM-1) in rats subjected to glycerol-induced AKI. **A:** Representative Western blot showing ICAM-1 protein expression in kidneys of untreated and glycerol-treated rats ($n = 4$) with or without suramin over a time course. **B:** Densitometric analysis of renal ICAM-1 protein expression in untreated, glycerol and suramin intervention after glycerol-treated groups over the time course. Data were normalized by GAPDH which served as internal control. Data are expressed as mean ± SE (n = 4). ***** Statistically significant from respective 0 h controls. # Statistically significant from glycerol and suramin-treated rats at the corresponding time point ($p \leq 0.05$).

Representative photomicrographs of ICAM-1-stained kidney sections from rats that were untreated (**C**); treated with glycerol alone, at 72 h (**D**); subjected to suramin-intervention after glycerol, at 72 h (**E**). ICAM-1 positive staining was observed on the basement membranes of

proximal tubules (arrows). All fields were chosen from the outer stripe of the outer medulla (OSOM). Original magnification, 200 X.

Fig. 8. Effect of delayed administration of suramin on renal pro-inflammatory cytokines in rats subjected to glycerol-induced AKI. Messenger RNA expression profile in kidneys of untreated and glycerol-treated rats (n = 4) with or without suramin over a time course and quantitative PCR was performed using primers for **A.** TNFα; **B.** IL-6; **C.** IL-1β; **D.** MCP-1. Tubulin was used as the internal control. Data are expressed as mean ± SE (n = 4). ***** Statistically significant from respective 0 h controls. # Statistically significant from glycerol alone-treated rats at the corresponding time point ($p \leq 0.05$).

Fig. 9. Effect of delayed administration of suramin on renal phospho-p65 in rats subjected to glycerol-induced AKI. **A:** Representative Western blot showing phospho-p65 protein expression in kidneys of untreated and glycerol-treated rats (n = 4) with or without suramin over a time course. **B:** Densitometric analysis of renal phospho-p65 protein expression in untreated, glycerol and suramin intervention after glycerol-treated groups over the time course. Data were normalized by GAPDH which served as internal control. Data are expressed as mean ± SE (n = 4). ***** Statistically significant from respective 0 h controls. # Statistically significant from glycerol alone-treated rats at the corresponding time point ($p \leq 0.05$).

Fig. 10. Effect of delayed administration of suramin on renal hemeoxygenase-1 (HO-1) and transforming growth factor-β_1 (TGF-β_1) in rats subjected to glycerol-induced AKI. Representative Western blot showing HO-1 (**A**) and TGF-β_1 (**C**) protein expression in kidneys of untreated and glycerol-treated rats (n = 4) with or without suramin over a time course. Densitometric analysis of renal HO-1 (**B**) and TGF-β_1 (**D**) protein expression in

untreated, glycerol and suramin intervention after glycerol-treated groups over the time course. Data were normalized by GAPDH, which served as internal control. Data are expressed as mean ± SE (n = 4). ***** Statistically significant from respective 0 h controls. @ Statistically significant from glycerol and suramin-treated rats at the corresponding time point. # Statistically significant from glycerol alone-treated rats at the corresponding time point ($p \leq 0.05$).

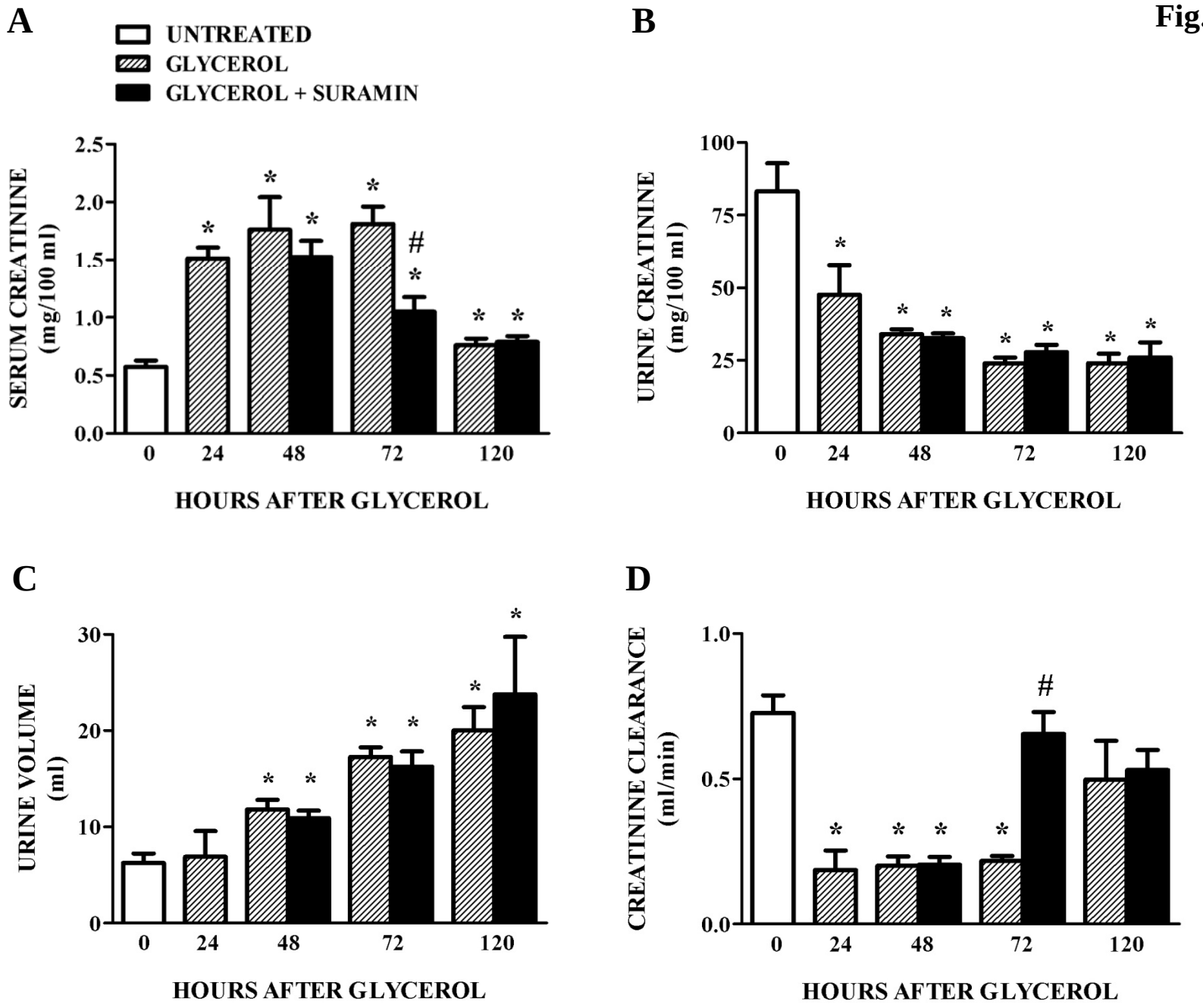
Fig. 11. Effect of delayed administration of suramin on renal proximal convoluted tubular epithelial cell proliferating cell nuclear antigen (PCNA) in rats subjected to glycerol-induced AKI. **A:** Representative Western blot showing PCNA protein expression in kidneys of untreated and glycerol-treated rats (n = 4) with or without suramin over a time course. **B:** Densitometric analysis of renal PCNA protein expression in untreated, glycerol and suramin intervention after glycerol-treated groups over the time course. Data were normalized by GAPDH which served as internal control. Data are expressed as mean ± SE (n = 4).

***** Statistically significant from respective 0 h controls. # Statistically significant from glycerol alone-treated rats at the corresponding time point ($p \leq 0.05$).

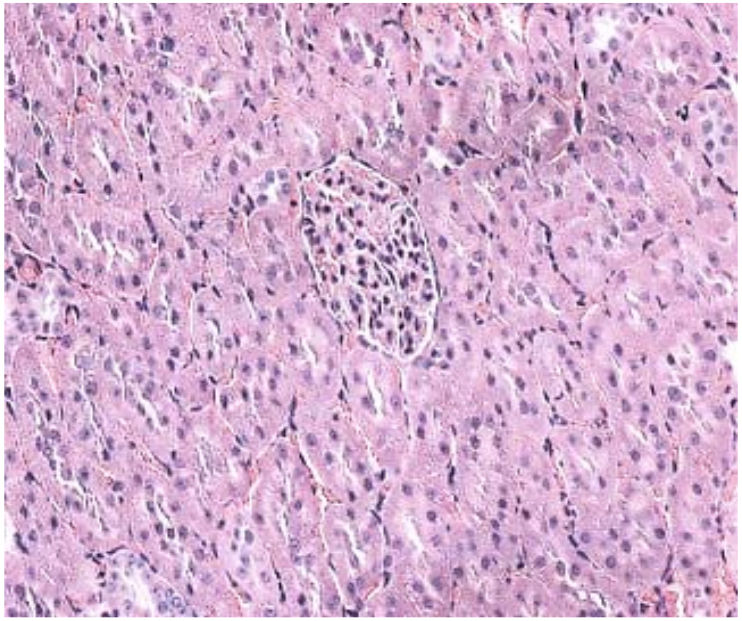
Representative photomicrographs of PCNA-stained kidney sections from rats that were untreated (**C**); treated with glycerol alone, at 72 h (**D**); subjected to suramin-intervention after glycerol, at 72 h (**E**). PCNA positive staining (intense brown) was observed in cells that are lining the proximal tubules (arrows). All fields were chosen from the outer stripe of the outer medulla (OSOM). Original magnification, 200 X.

.

Fig. 1


A
UNTREATED
GLYCEROL
GLYCEROL + SURAMIN
SERUM CREATININE (mg/100 ml)
2.5
2.0
1.5
1.0
0.5
0.0
0
24
48
72
120
HOURS AFTER GLYCEROL
B
URINE CREATININE (mg/100 ml)
100
75
50
25
0
HOURS AFTER GLYCEROL
C
URINE VOLUME (ml)
30
20
10
0
HOURS AFTER GLYCEROL
D
CREATININE CLEARANCE (ml/min)
1.0
0.5
0.0
HOURS AFTER GLYCEROL

A. UNTREATED



B. 72 H AFTER GLYCEROL

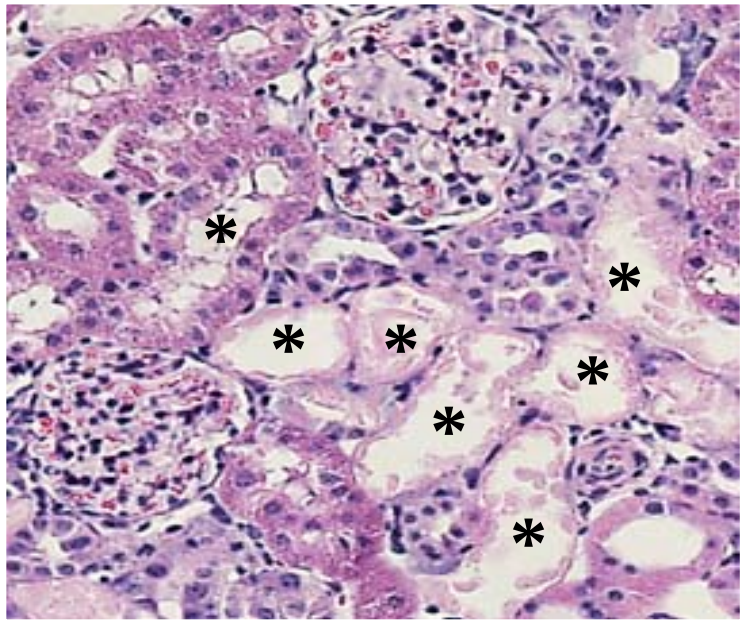


Fig. 2

C. 72 H AFTER GLYCEROL + SURAMIN

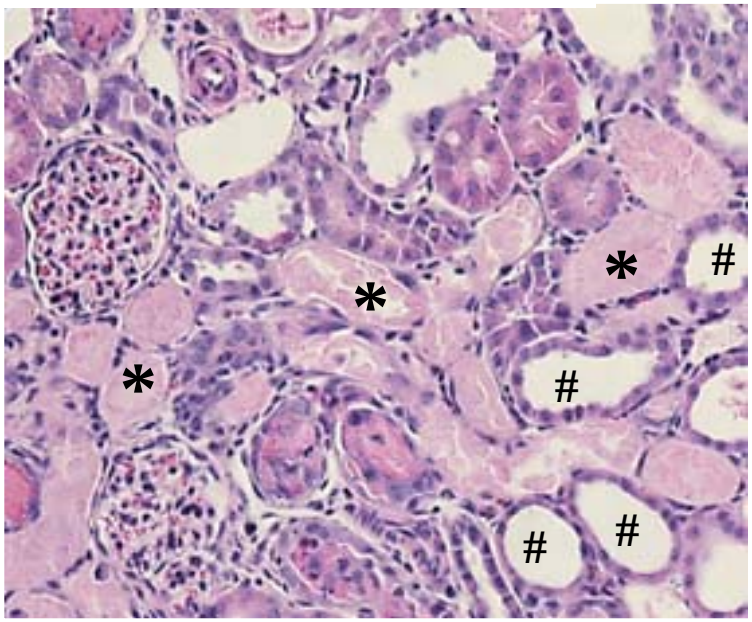
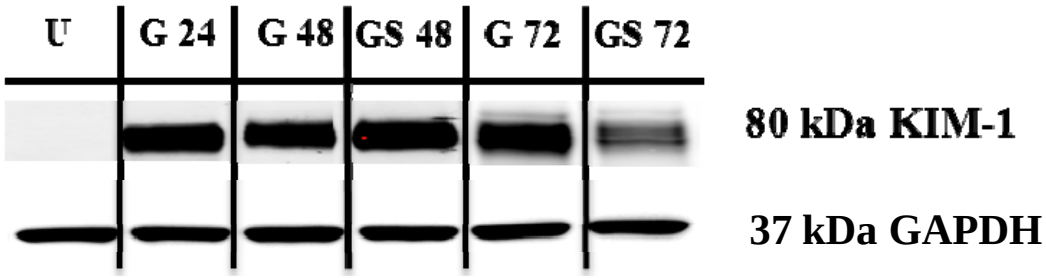


Fig. 3

A



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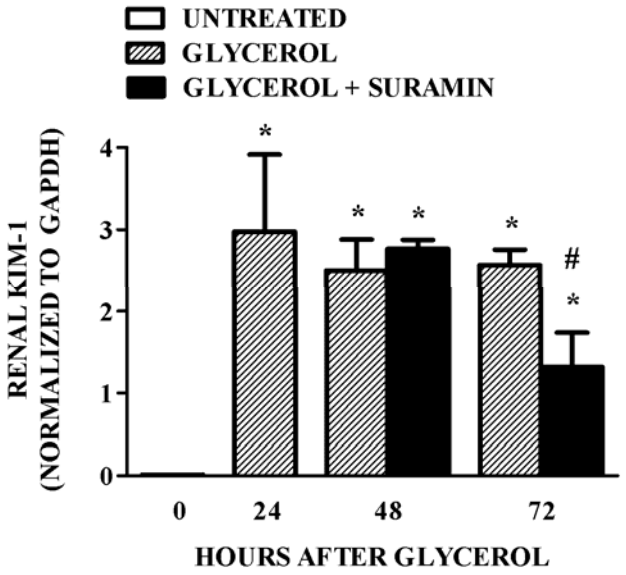

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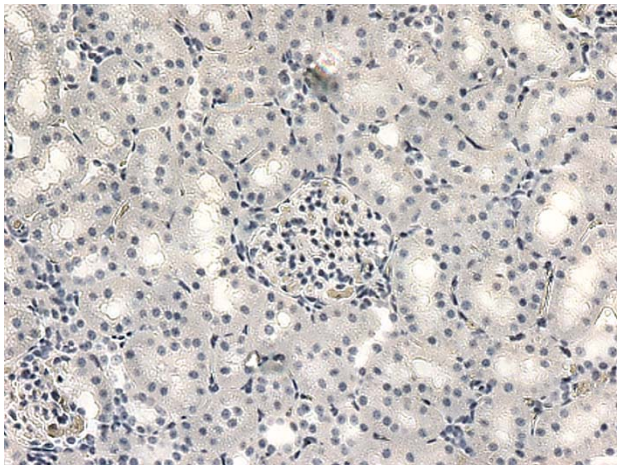
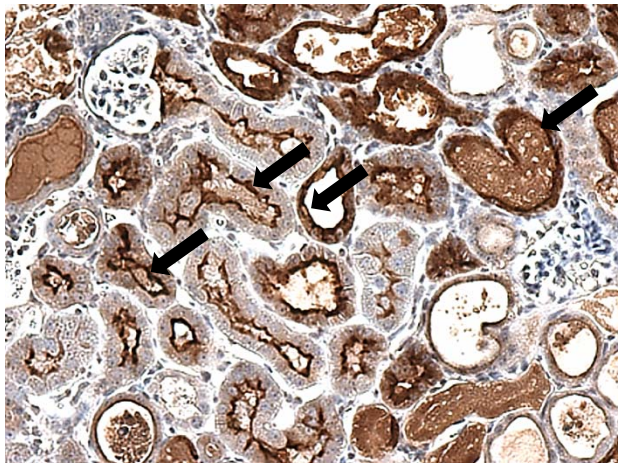


D. 72 H AFTER GLYCEROL



E. 72 H AFTER GLYCEROL + SURAMIN

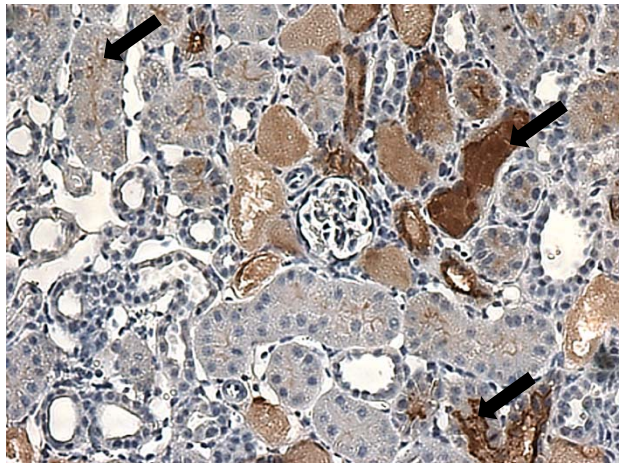
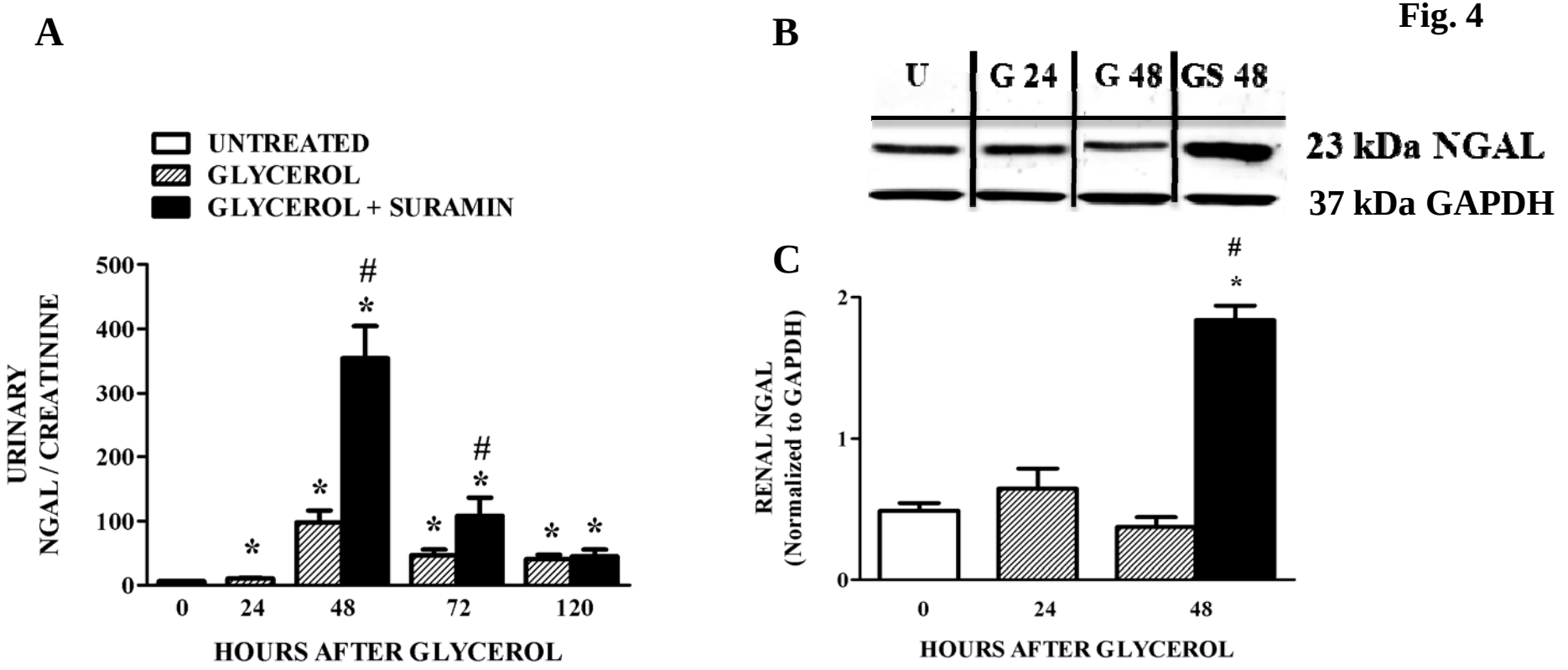


Fig. 4



D. UNTREATED

E. 48 H AFTER GLYCEROL

F. 48 H AFTER GLYCEROL + SURAMIN

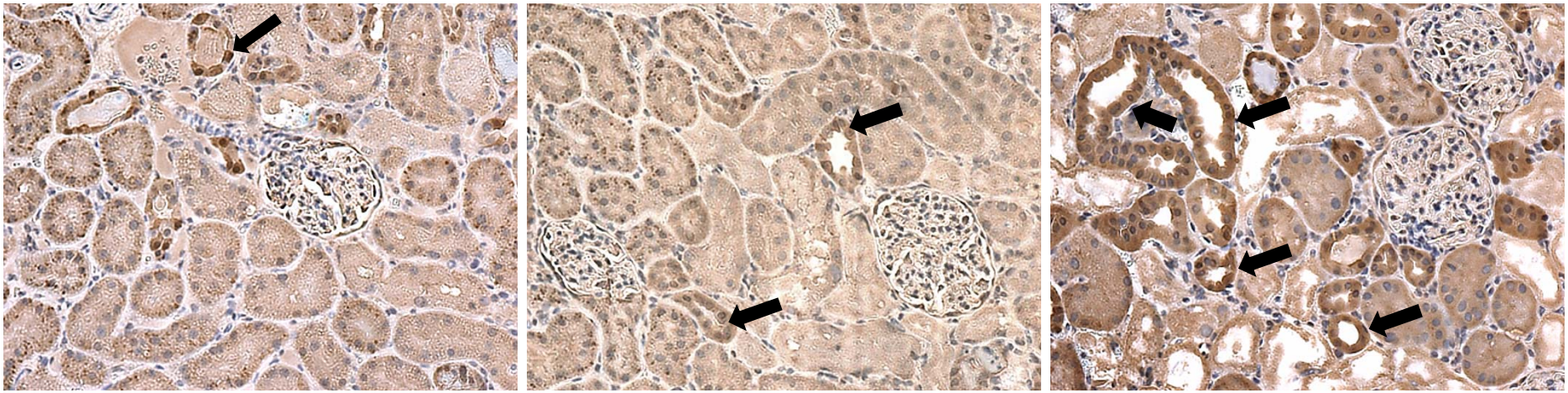
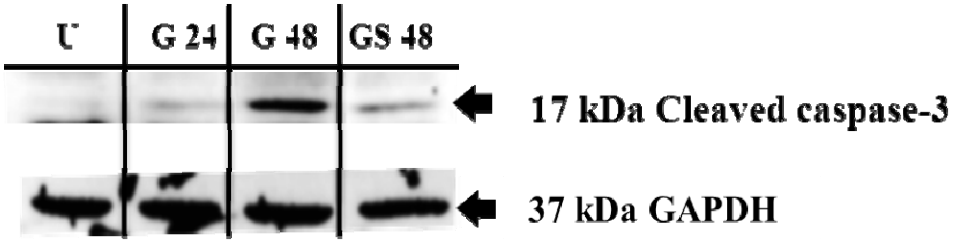


Fig. 5

A


U
G 24
G 48
GS 48
17 kDa Cleaved caspase-3
37 kDa GAPDH

B

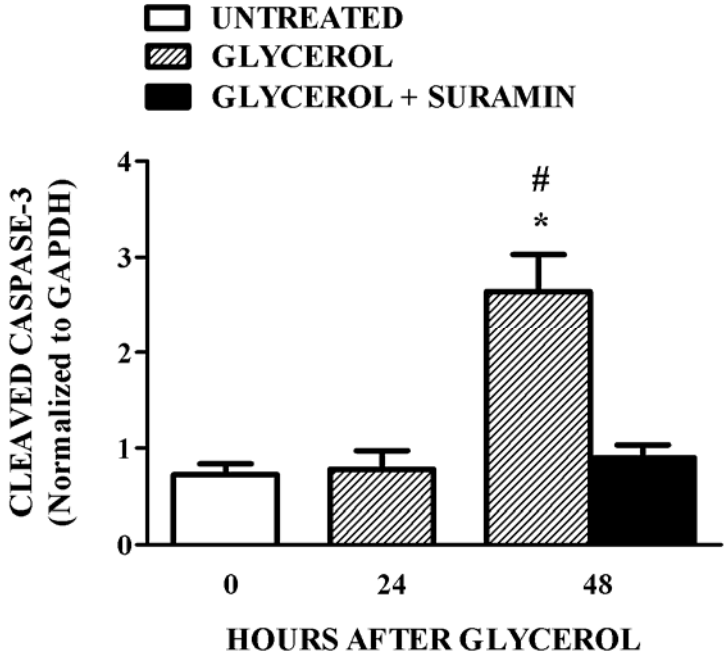
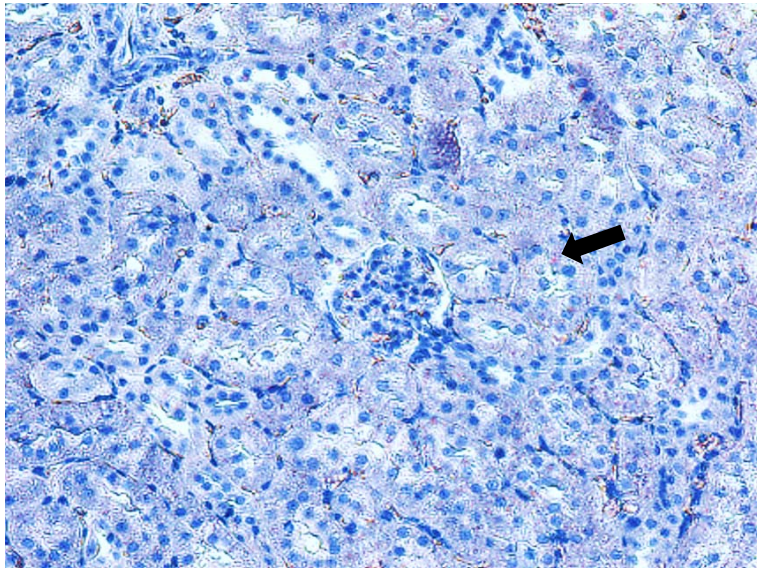
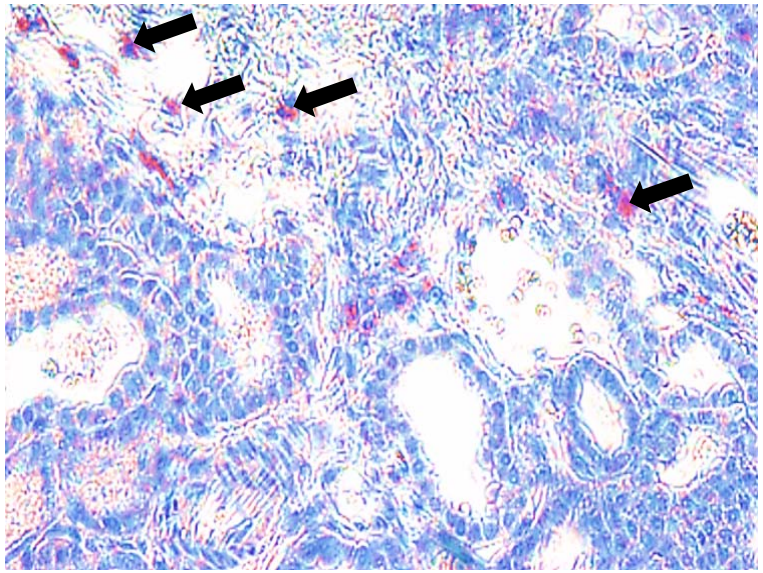

UNTREATED
GLYCEROL
GLYCEROL + SURAMIN
CLEAVED CASPASE-3
(Normalized to GAPDH)
0
1
2
3
4
#
*
0
24
48
HOURS AFTER GLYCEROL

Fig. 6

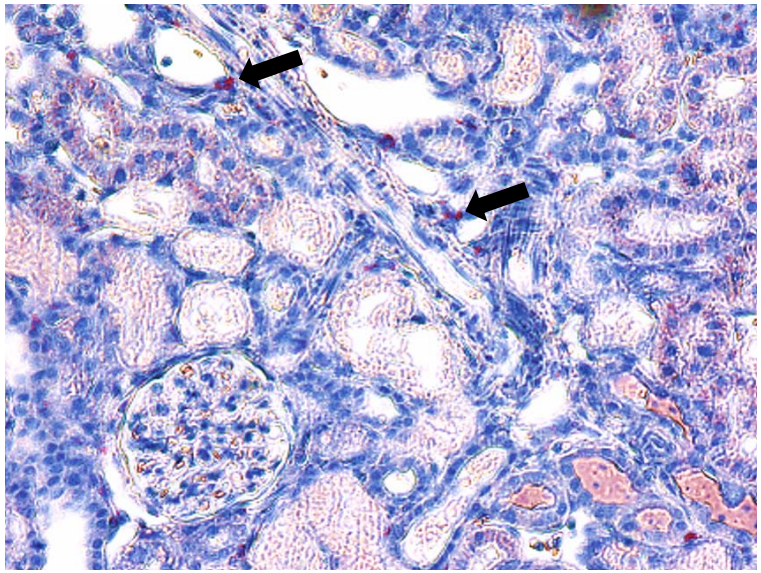
A. UNTREATED



B. 72 H AFTER GLYCEROL



C. 72 H AFTER GLYCEROL + SURAMIN



D

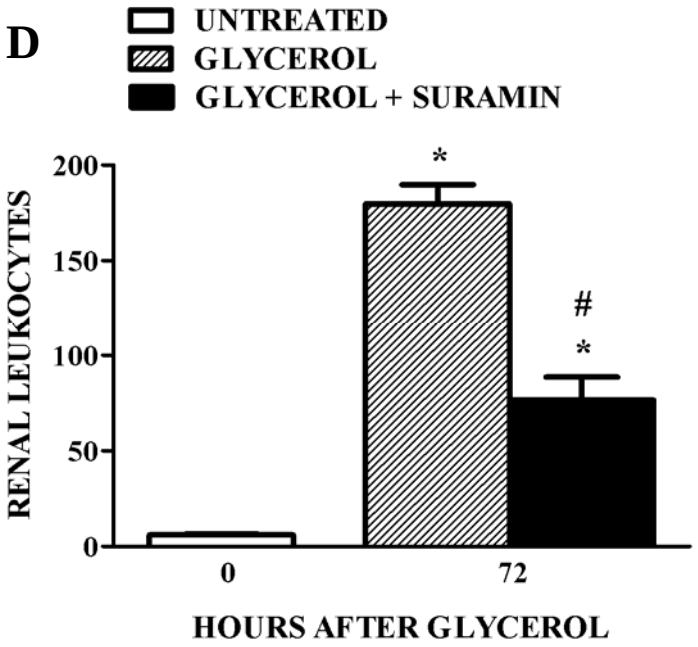
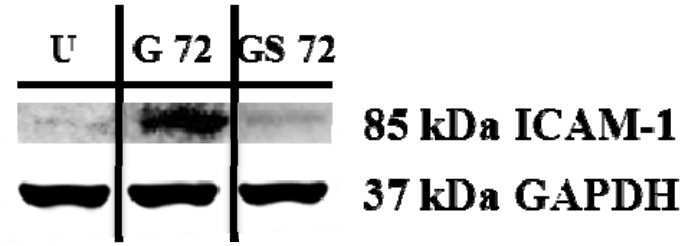
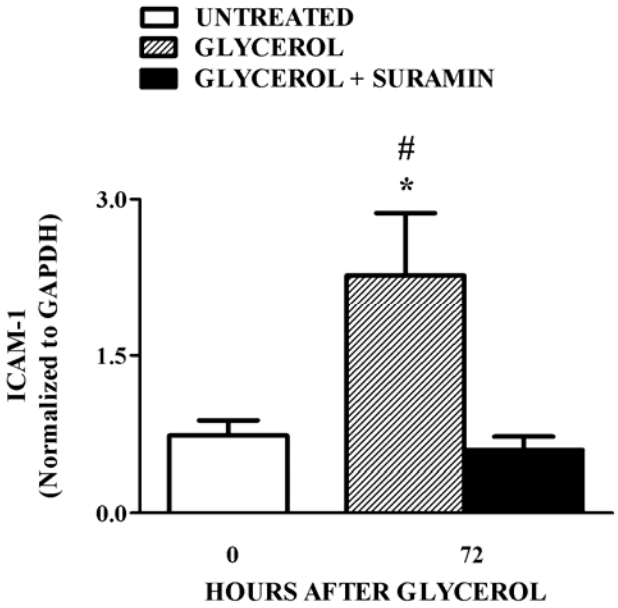


Fig. 7

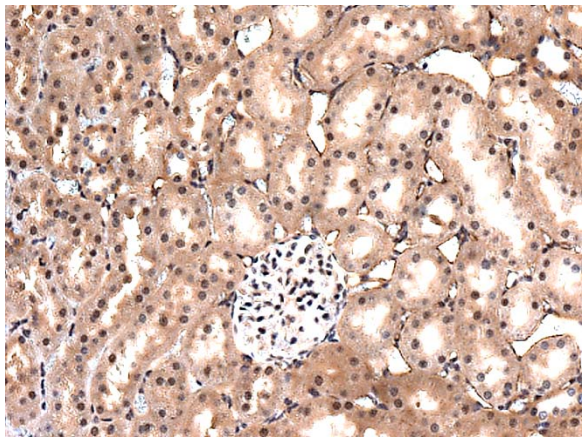
A



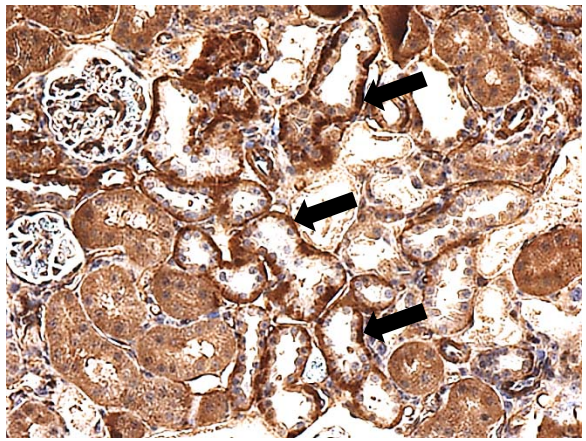
B



C. UNTREATED



D. 72 H AFTER GLYCEROL



E. 72 H AFTER GLYCEROL + SURAMIN

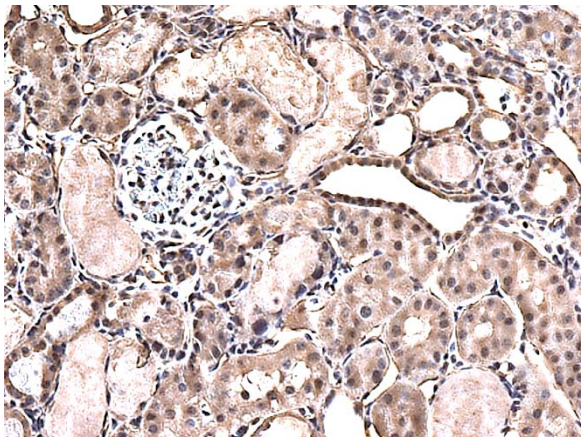


Fig. 8

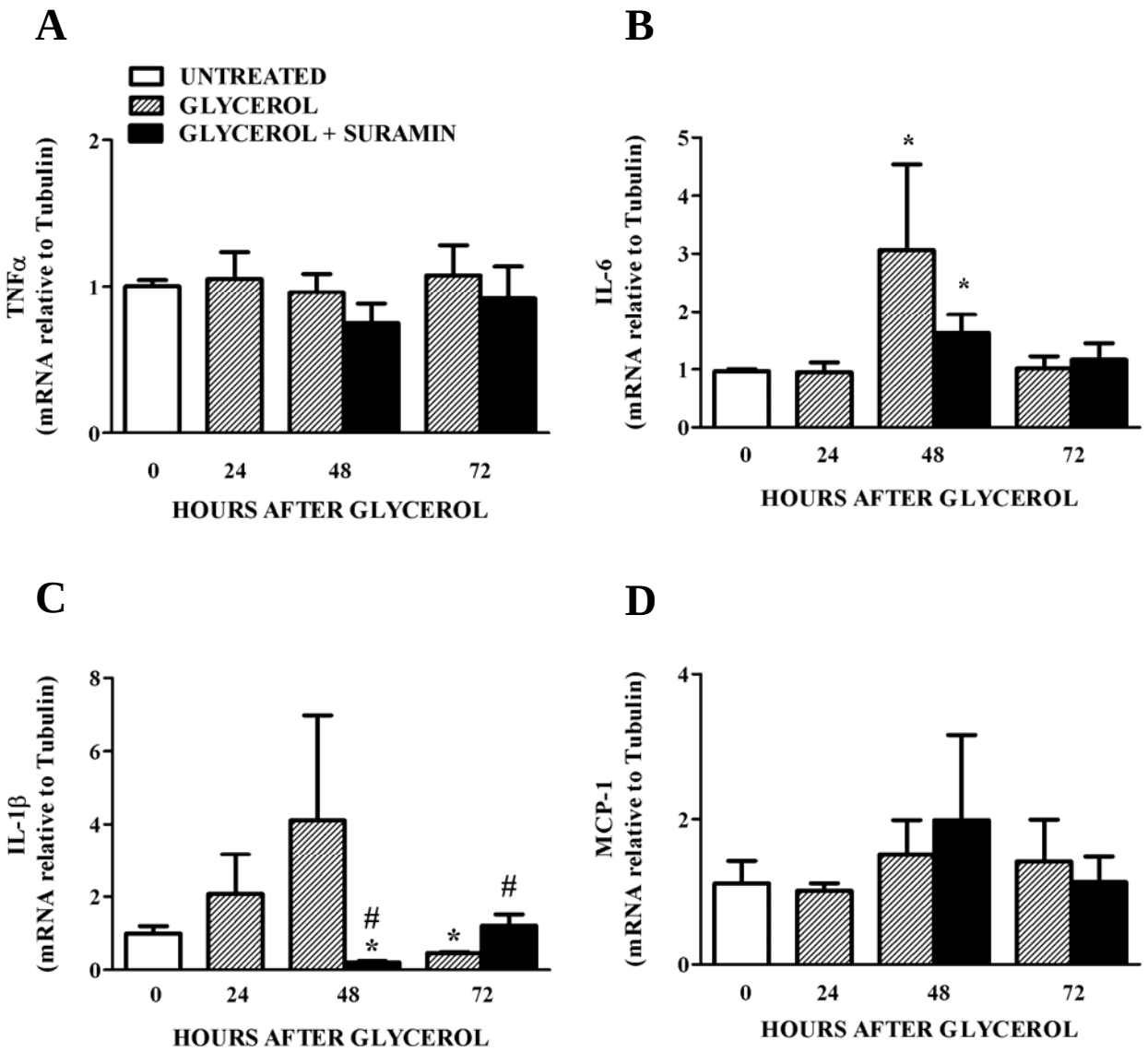

A
UNTREATED
GLYCEROL
GLYCEROL + SURAMIN
TNFα
(mRNA relative to Tubulin)
0
1
2
0
24
48
72
HOURS AFTER GLYCEROL
B
IL-6
(mRNA relative to Tubulin)
0
1
2
3
4
5
*
*
0
24
48
72
HOURS AFTER GLYCEROL
C
IL-1β
(mRNA relative to Tubulin)
0
2
4
6
8
#
*
*
#
0
24
48
72
HOURS AFTER GLYCEROL
D
MCP-1
(mRNA relative to Tubulin)
0
2
4
0
24
48
72
HOURS AFTER GLYCEROL

Fig. 9

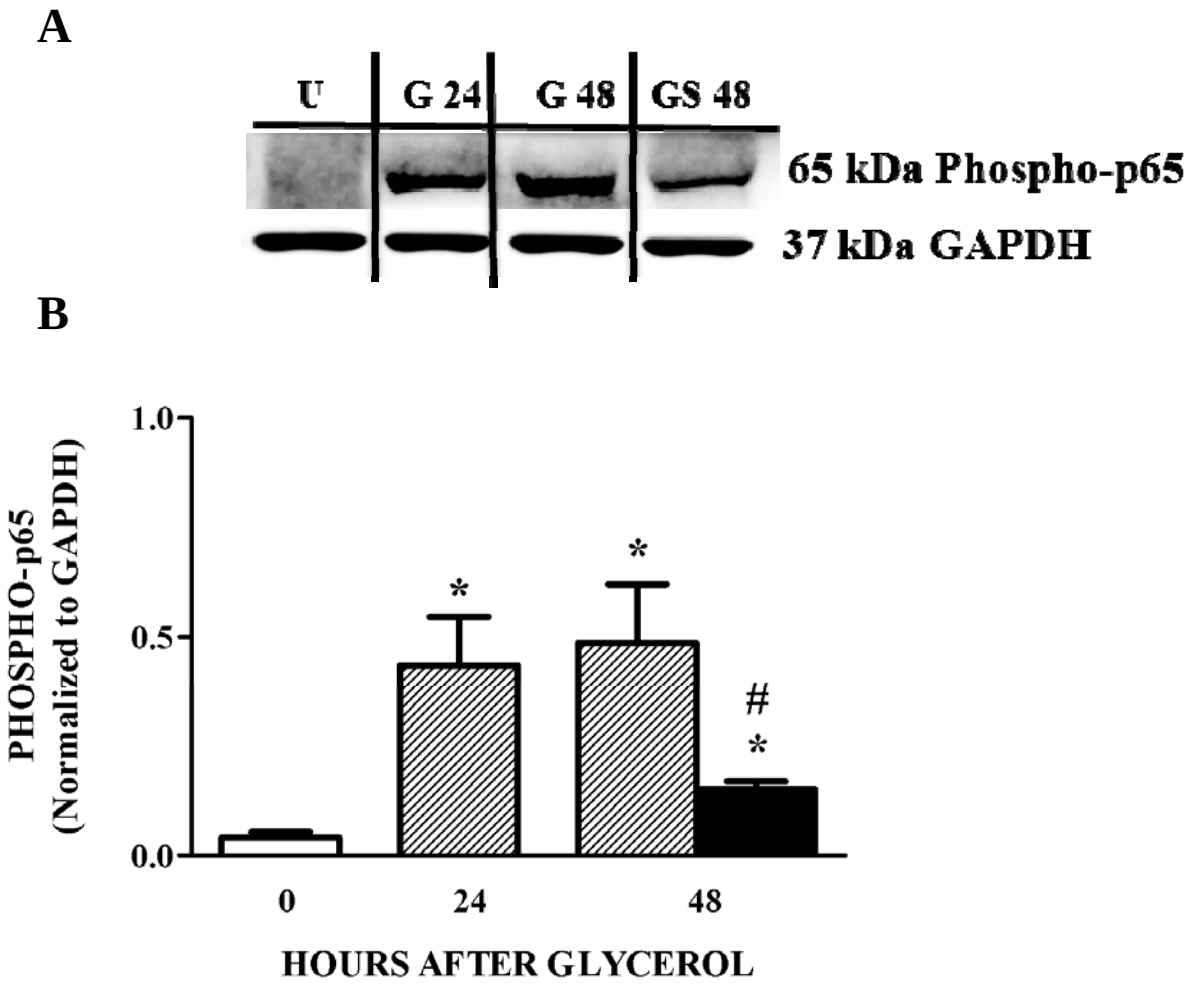
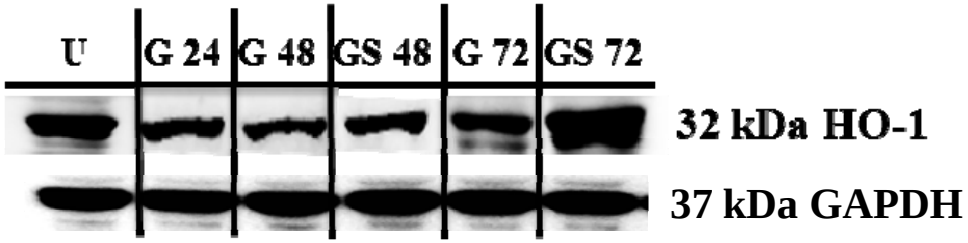
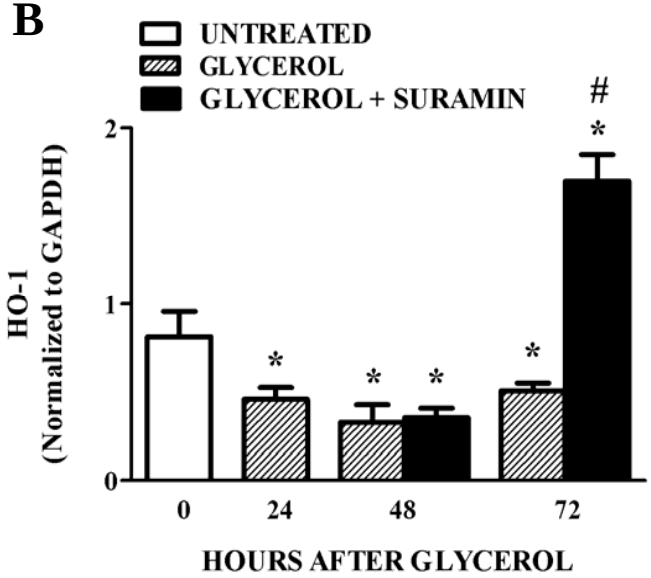

A
U
G 24
G 48
GS 48
65 kDa Phospho-p65
37 kDa GAPDH
B
PHOSPHO-p65
(Normalized to GAPDH)
1.0
0.5
0.0
*
*
#
*
0
24
48
HOURS AFTER GLYCEROL

Fig. 10

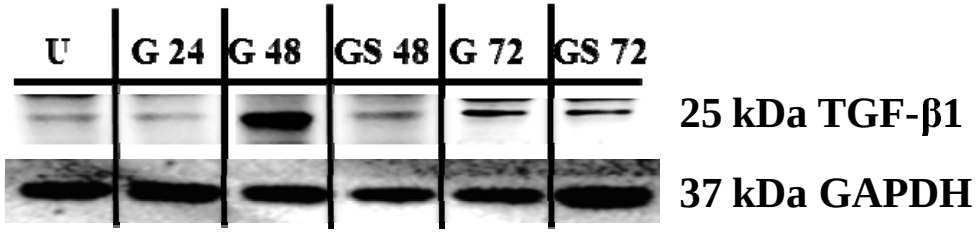
A


U
G 24
G 48
GS 48
G 72
GS 72
32 kDa HO-1
37 kDa GAPDH

B


UNTREATED
GLYCEROL
GLYCEROL + SURAMIN
HO-1
(Normalized to GAPDH)
0
1
2
0
24
48
72
HOURS AFTER GLYCEROL
*
#

C


U
G 24
G 48
GS 48
G 72
GS 72
25 kDa TGF-β1
37 kDa GAPDH

D

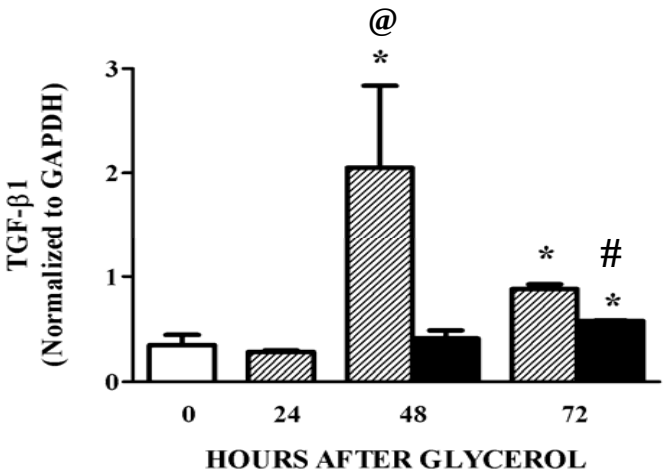
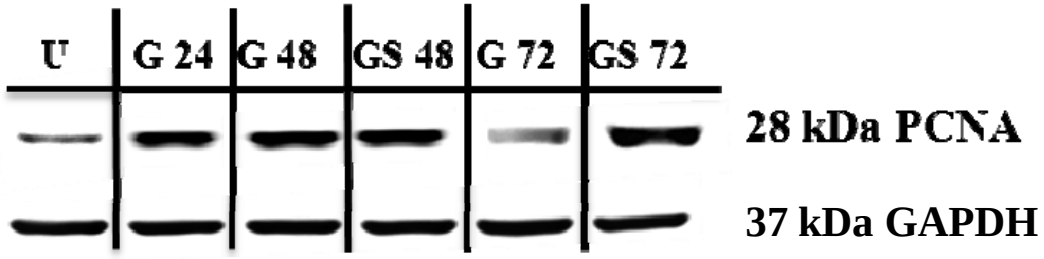
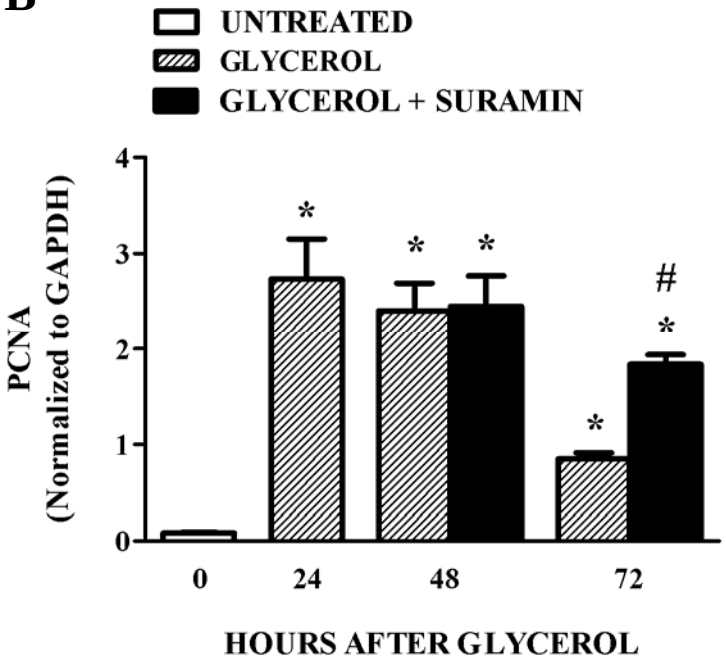

TGF-β1
(Normalized to GAPDH)
0
1
2
3
0
24
48
72
HOURS AFTER GLYCEROL
@
*
#

Fig. 11

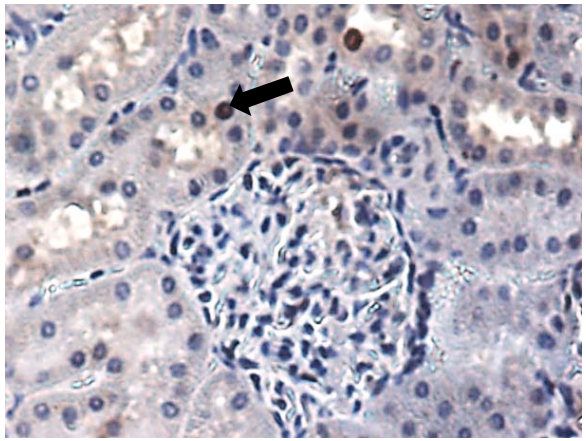
A



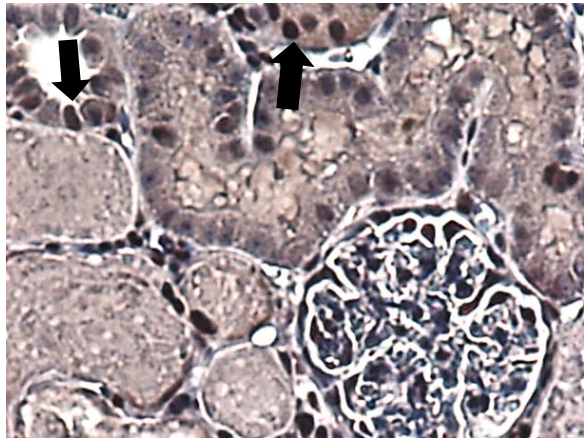
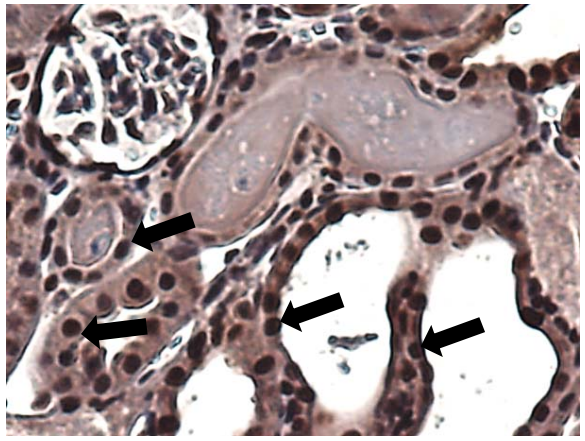
B



C. UNTREATED



D. 72 H AFTER GLYCEROL



E. 72 H AFTER GLYCEROL + SURAMIN