NEPHROPROTECTIVE EFFECT OF METFORMIN AGAINST GLYCEROL-INDUCED ACUTE KIDNEY INJURY IN RATS

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ABSTRACT

Background:

Rhabdomyolysis-associated acute kidney injury (AKI) is a serious life-threatening condition. Therefore, more effective strategies are needed for its prevention. The pathophysiological process of rhabdomyolysis-associated AKI involves oxidative stress, inflammation and apoptosis. Metformin (MF) has been known to possess antioxidant, anti-inflammatory and antiapoptotic properties. This study explored the possible effect of pretreatment with metformin on the development of glycerol-induced-AKI in rats.

Objective:

The objective of this study was to investigate the potential protective effects of metformin against glycerol-induced AKI in rats, so as to provide an experimental basic for the use of metformin in the clinical prevention of rhabdomyolysis-induced AKI.

The renoprotective effect, anti-oxidative, anti-inflammative and anti-apoptotic action on the rat model were evaluated in vivo.

Materials and methods:

Forty rats were randomly divided into four equal groups. Group 1 served as the control, group 2 was given 50% glycerol (10 mL/kg, i.m.), group 3 was given glycerol after 10 days of MF treatment (100 mg/kg/day, p.o.), and group 4 was given MF alone for 10 days 100 (mg/kg/day, p.o.). Renal function was monitored by serum creatinine (SCr), Blood urea nitrogen (BUN), and histologic analysis. Moreover, oxidative stress was monitored by kidney tissue superoxide dismutase (SOD), and catalase (CAT) activities, inflammation was monitored by interleukin 6 (IL-6) evaluation, and finally apoptosis was monitored by measuring active caspase 3 content.
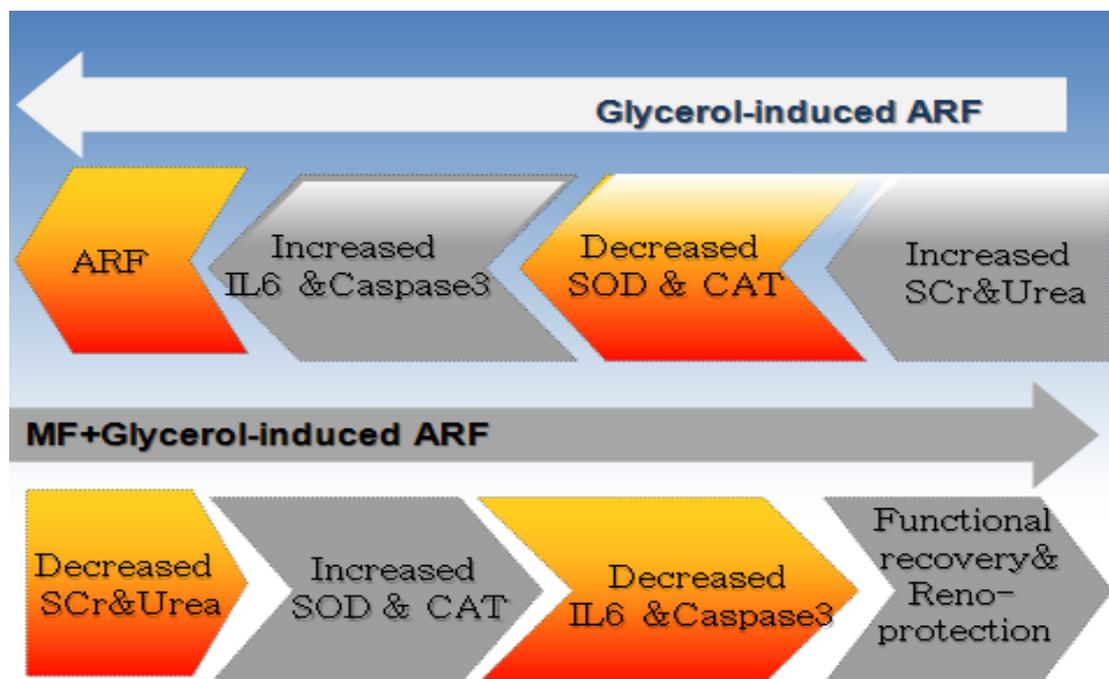
Results:

Glycerol treatment resulted in an increase in the mean histologic damage score, SCr, BUN, kidney tissue IL-6, caspase 3 and a decrease in kidney tissue SOD and CAT activities. All these factors were significantly improved by pretreatment with MF.

Conclusions:

Pretreatment with MF ameliorated renal dysfunction in glycerol-induced rhabdomyolysis by inhibiting oxidative stress, inflammatory response and apoptosis.

Graphical abstract

Glycerol-induced ARF

ARF | Increased IL6 &Caspase3 | Decreased SOD & CAT | Increased SCr&Urea

MF+Glycerol-induced ARF

Decreased SCr&Urea | Increased SOD & CAT | Decreased IL6 &Caspase3 | Functional recovery& Reno-protection

Metformin protected the kidney by targeting various pathways:

Treatment with MF reduced progression of glycerol-induced AKI by targeting multiple pathways. MF reduces oxidative stress, inflammation and apoptosis

Key words: Rhabdomyolysis, glycerol, AKI, MF

Introduction

Rhabdomyolysis is a clinical condition caused by an acute disruption of skeletal muscle due to physical or chemical damage from a crushing injury, exhaustive exercise, surgery, temperature extremes, certain types of drugs or toxins (Huerta-Alardín et al., 2005). The damaged skeletal muscle results in the leakage of myoglobin and other intracellular proteins into the circulation causing myoglobinuria, and if this is severe enough it can result in AKI. In general, about 10–50% of patients with rhabdomyolysis develop AKI (Ward, 1988), accounting for 7 to 10% of all AKI cases (Bagley et al., 2007).

The experimental model for rhabdomyolysis is produced by glycerol treatment which disrupts skeletal muscle and develops a model that clinically mimics the rhabdomyolysis-induced AKI in humans (Singh et al., 2012).

Different mechanisms have been proposed for the pathology of glycerol-induced AKI, namely oxidative stress, inflammation and apoptosis. The release of myoglobin

from muscle cells results in myoglobin concentration along the renal tubules where it leads to the uncontrolled leakage of reactive oxygen species (ROS) ending with tubular injury (Zager and Foerder, 1992). In addition, the rise in the number of macrophages (Homsi et al., 2009) and released inflammatory cytokines such as tumor necrosis factor-α (TNF-α) are typical features in glycerol-induced AKI (Shulman et al., 1993). IL-6 is produced in copious amounts by endothelial cells in response to TNF-α (Shalaby et al., 1989), contributing in part toward the generation of renal injury (Nechemia-Arbely et al., 2008). Furthermore, it was reported that apoptosis also participates in important pathogenic mechanisms in glycerol-induced AKI (Homsi et al., 2006). Therefore, targeting oxidative stress, inflammation and apoptosis is a reasonable approach for the prevention or treatment of rhabdomyolysis-induced AKI.

Metformin (MF) is a drug primarily used in the treatment of Diabetes Mellitus type II where it suppresses glucose production by the liver. Various studies revealed that MF is more than a simple antidiabetic agent. In fact, for an equivalent effect on glycemic control after a long time of treatment, MF was found to be conspicuously superior to other therapeutic measures for reducing vessel diseases and all-cause related mortality (Gheissari et al., 2012). By improving glycoxidative, inflammatory, and fibrotic renal damage markers, MF was suggested to play a key role in the prevention of diabetic nephropathy (Louro et al., 2011). Moreover, MF showed antioxidant properties resulting in reductions in both oxidative damage accumulation and chronic inflammation (Martin-Montalvo et al., 2013; Nasri et al., 2013). Recently, MF was found to possess anti-inflammatory and antiapoptotic features on renal ischemia/reperfusion injury in rats (Wang et al., 2015).

Despite promising preclinical data on the renoprotective effects of MF against various insults such as diabetic nephropathy and gentamicin induced-AKI (Alhaider et al., 2011; Amini et al., 2012), there is lack of information describing the renal benefits of MF for nondiabetic patients who are at high risk of rhabdomyolysis-induced AKI. Thus, experimental studies are needed to elaborate possible renoprotective benefits of MF. With this background, in the present study, a glycerol-induced model of AKI is used to elucidate the potential protective role of MF against rhabdomyolysis-induced AKI and its underlying mechanisms.

The objective of our study was to investigate the potential protective effect of MF against rhabdomyolysis-induced AKI.

Materials and Methods

The study was conducted in accordance with ethical procedures and policies approved by the ethics committee of faculty of pharmacy, girls, Al-Azhar University.

Animals

Forty adult male albino rats weighing 200-250g were purchased from the animal house of El Nile Co. for pharmaceutical, El Amyria, Cairo, Egypt The animals were kept at controlled environmental conditions in terms of constant temperature (24±1°C), humidity (60±10%), and a 12/12 h light/dark cycle. They were acclimatized for one week before any experimental procedures and were allowed standard rat chow and water *ad libitum*.

Drugs and chemical

Metformin was obtained from Sigma-Aldrich (St.Louis, MO, USA) and was freshly prepared as an aqueous solution to be given as a single daily oral dose of 100 mg/kg/day (Morales et al., 2010). Glycerol was purchased from El-Gomhuria Co. Cairo, Egypt. All other chemicals were of highest purity and analytical grade.

Induction of glycerol-induced AKI

Induction of AKI was accomplished by intramuscular administration of a single dose of 50% hypertonic glycerol (10 ml/kg), The required amount of glycerol was administered as a deep *im* injection equally distributed to both hind legs (Singh et al., 2012).

Experimental design

Forty male Sprague-Dawley rats, weighing 200-250 g were used in the present study. They were divided randomly into four equal groups of 10 animals each. The animals were allowed free access to food, but deprived of drinking water for 24 h before glycerol injection.

Group 1: Normal control group. The animals received sterile saline orally (100mL/kg/day) for 10 days, then were given saline (10 mL/kg, i.m.) on the tenth day. Half the dose was administered to each hind limb muscle.

Group 2: Glycerol group. The animals received saline orally (100mL/kg/day) for 10 days, then were given hypertonic glycerol (50%, v/v in sterile saline) (10 mL/kg, i.m.) on the tenth day. Half the dose was administered to each hind limb muscle.

Group 3: MF/glycerol group. The animals received metformin orally (100 mg/kg/day) for 10 days, then were given hypertonic glycerol (50%, v/v in sterile saline) (10 mL/kg, i.m.) on the tenth day. Half the dose was administered to each hind limb muscle.

Group 4: MF control group. The animals received metformin orally (100 mg/kg/day) for10 d, then were given saline (10 mL/kg, i.m.) on the tenth day. Half the dose was administered to each hind limb muscle.

The last dose of MF was given one hour before glycerol injection (Singh et al., 2004).

After glycerol injection rats were kept for 48 h with free access to water and diet. Then all rats were anesthetized with 2% pentobarbital sodium (40 mg/kg, i.p). After complete anesthesia, a midline abdominal incision was performed, and then their blood was collected *via* intracardiac puncture. Blood samples were centrifuged after 30 min (4000 *g* for 10 min at 4°C), and samples were stored at −80°C until assay. After blood collection, the kidneys were harvested. The left kidney was frozen at −80°C for further enzymatic analysis; kidneys of 2 rats were preserved in 10% formaline saline for the histopathological study. Kidney homogenates were prepared as 20% homogenate in normal saline.

Assessment of renal function

Serum samples were examined for blood urea nitrogen using standard diagnostic kits (QuantiChromTM Urea Assay Kit (DIUR-500), Hayward, USA) and creatinine (QuantiChromTM Creatinine Assay Kit (DICT-500), Hayward, USA) in an autoanalyser (ADIVA 1650, Bayer, Japan).

Oxidative stress index

Kidney tissue superoxide dismutase (SOD) and catalase (CAT) activities were measured using OxiSelect™ Superoxide Dismutase Activity assay (CELL BIOLABS, Inc., San Diego, USA) and EIAab™ Rat Catalase (CAT) ELISA kit (Elaab Science Co., Wuhan, China) following the instructions from the manufacturer.

Inflammation and apoptosis index

Kidney tissue Interleukin 6 (IL-6) and active caspase 3 contents from the harvested and frozen kidney tissue were measured using (RayBio® Rat IL-6 ELISA Kit (RayBiotech, Inc. Norcross, GA) and Rat Caspase 3 ELISA Kit (Cusabio Biotech, Hubei, China) respectively, following the instructions from the manufacturer.

Kidney morphologic studies

Histopathological studies were done according to the method of (Levison, 1997)**.** Autopsy samples were taken from the kidney of rats in different groups and fixed in 10% formaline saline for twenty four hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin & eosin stain for routine examination through the light electric microscope.

Statistical analysis:

The date were expressed as mean ± SEM of 8 animals and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey- Kramer for multiple comparisons at $p < 0.05$. A computer program (graph pad instat, version 3) was used for statistical analysis. Another computer program, Graph Pad Prism 5.0, San Diego, CA, was used for drawing figures.

RESULTS

The effects of MF on SCr and BUN levels

The data for SCr and BUN showed that glycerol administration elevated both levels significantly compared to the normal control group (P< 0.05), while pretreatment with MF decreased both levels significantly (P< 0.05) as illustrated in fig.1A and B.

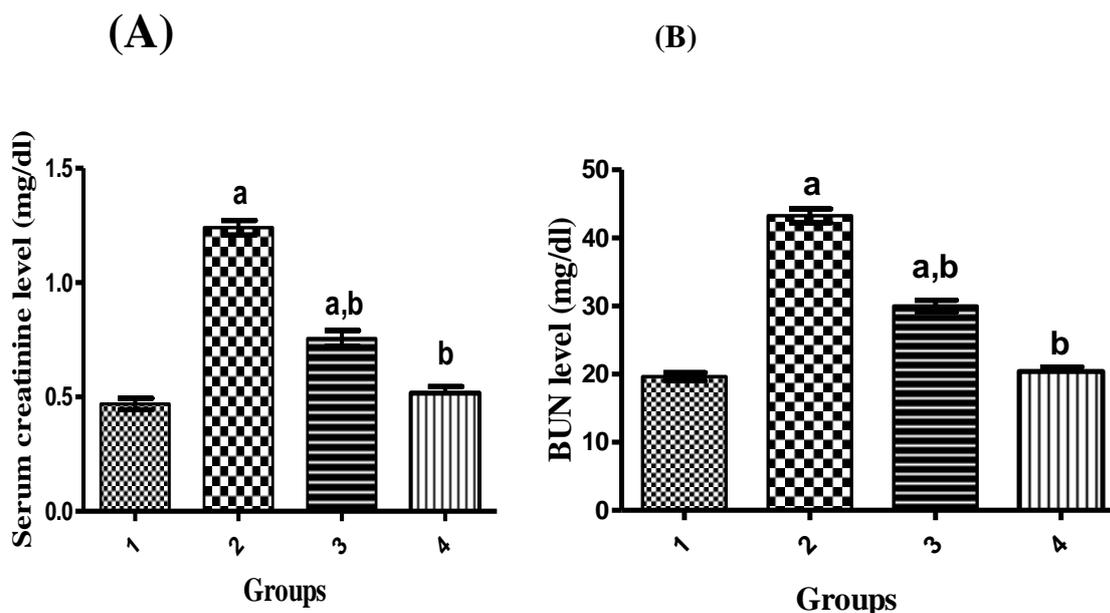**(A)**   **(B)**

Fig. 1: Effect of MF on SCr (A) and BUN (B). Group I, normal control group; group II, glycerol treated group (10ml/kg. i.m.); group III, MF/glycerol group. MF (100mg/kg/day) for 10 day + glycerol; group IV, MF control group (100mg/kg/days) for 10 days. Data were expressed as mean ± SE (n= 8), The symbols (a) and (b) stand for significant difference from normal control ($P < 0.05$) and glycerol treated group ($P < 0.05$), respectively.

The effects of MF on antioxidative stress index

The data for homogenate level of both SOD and CAT showed significant decline of both activities after treatment with glycerol compared to the control group (P< 0.05). However, pretreatment with MF significantly raised both activities compared to glycerol treatment (P< 0.05) as illustrated in fig. 2A and B.

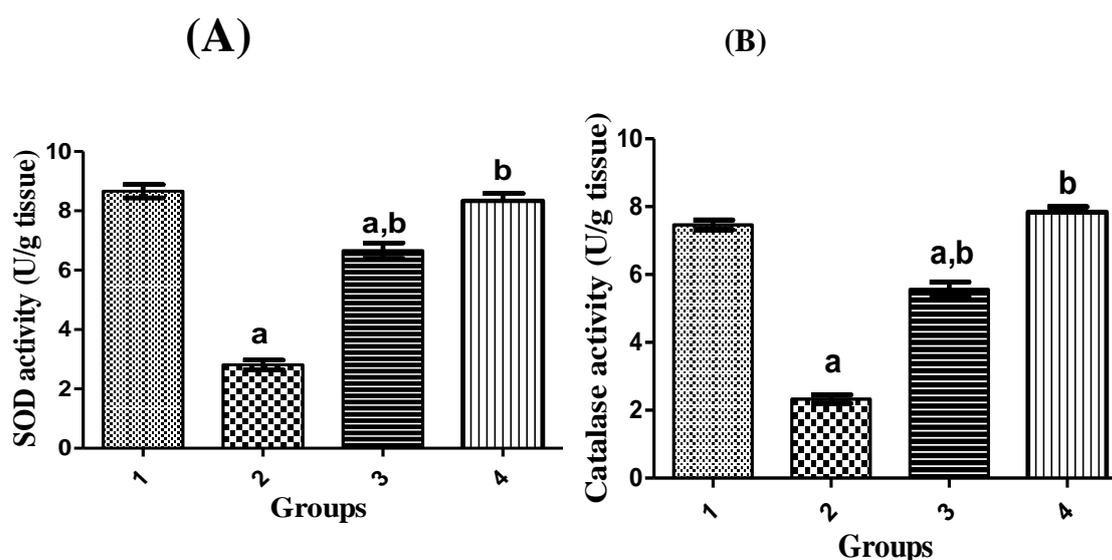**(A)**   **(B)**

Fig. 2: Effect of MF on SOD (A) and CAT (B). Group I, normal control group; group II, glycerol treated group (10ml/kg. i.m.); group III, MF/glycerol group. MF

(100mg/kg/day) for 10 day + glycerol; group IV, MF control group (100mg/kg/days) for 10 days. Data were expressed as mean ± SE (n= 8), The symbols (a) and (b) stand for significant difference from normal control ($P < 0.05$) and glycerol treated group ($P < 0.05$), respectively.

The effect of MF on inflammatory index

Interleukin-6 (IL-6) content showed significant elevation in glycerol treated group from the control group (P< 0.05), on the other hand group received Glycerol+ MF showed significant increase in IL-6 level compared with glycerol treated group (P< 0.05) as illustrated in fig. 3.
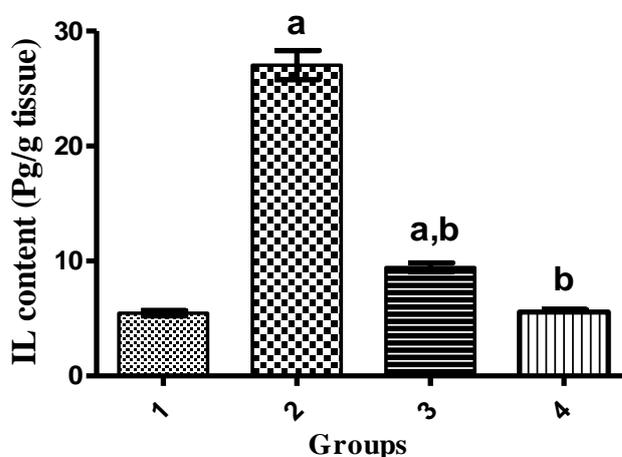

Fig. 3: Effect of MF on kidney tissue IL-6. Group I, normal control group; group II, glycerol treated group (10ml/kg. i.m.); group III, MF/glycerol group.   MF (100mg/kg/day) for 10 day + glycerol; group IV, MF control group (100mg/kg/days) for 10 days. Data were expressed as mean ± SE (n= 8), The symbols (a) and (b) stand for significant difference from normal control ($P < 0.05$) and glycerol treated group ($P < 0.05$), respectively.

The effects of MF on apoptosis

In glycerol treated group, active caspase 3 level showed significant increase from the corresponding control group (P< 0.05), while group received Glycerol+ MF showed significant decrease compared with glycerol treated group (P< 0.05) as illustrated in fig. 4.
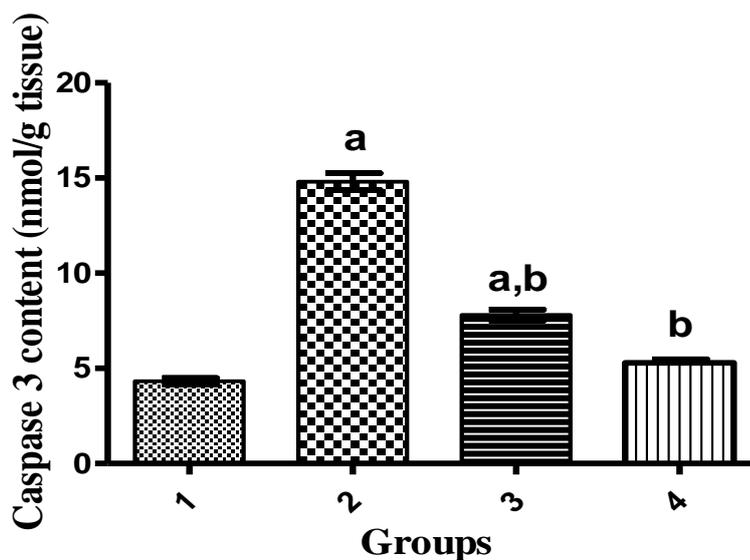
Fig. 4: Effect of MF on kidney tissue caspase 3. Group I, normal control group; group II, glycerol treated group (10ml/kg. i.m.); group III, MF/glycerol group. MF (100mg/kg/day) for 10 day + glycerol; group IV, MF control group (100mg/kg/days) for 10 days. Data were expressed as mean ± SE (n= 8), The symbols (a) and (b) stand for significant difference from normal control ($P < 0.05$) and glycerol treated group ($P < 0.05$), respectively.

Effect of MF on renal tubular injuryinduced by glycerol

The kidney sections of both normal control and positive control group exhibited normal renal architecture (Fig. 5A and B). In the glycerol group, the basic histologic abnormalities were coagulative necrosis, multiple eosinophillic cast formation, focal inflammatory cell infiltration (Fig. 5C and D). Compared with the glycerol group, tubular cell necrosis and cast formation partially decreased with MF pretreatment (Fig. 5E).
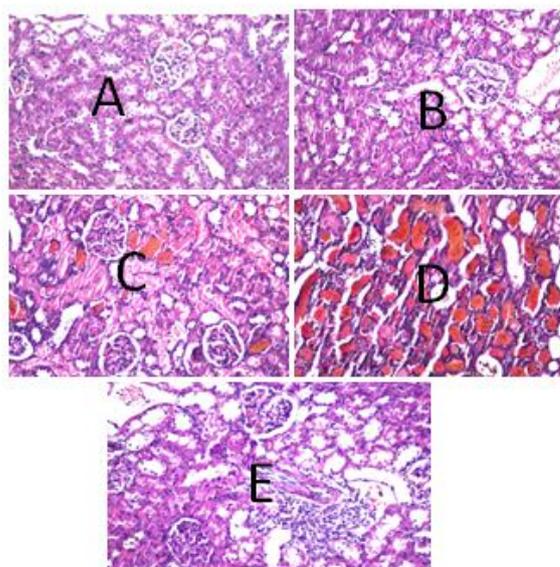

Fig. 5: Photomicrograph for kidney sections (original magnification×400). (A) normal control group. (B) MF control group. (C and D) Glycerol treated group. (E) MF/glycerol group.

Discussion

AKI is a serious life-threatening complication of rhabdomyolysis. Interventions to overcome this problem, as using mannitol and bicarbonate to preserve renal function, have limited clinical evidence. Therefore, new strategies that might alleviate rhabdomyolysis-induced AKI are needed. Several studies reveal encouraging features of metformin (MF), namely antioxidant, anti-inflammatory and antiapoptotic characteristics (Cahova et al., 2015; Singh et al., 2015; Wang et al., 2015).

In the present study, we investigated the potential influence of MF on rhabdomyolysis-induced AKI. Animals were deprived of drinking water before the i.m. injection of glycerol as hypovolaemia facilitates tubular precipitation of myoglobin casts and helps in accelerating renal damage. According to our data, glycerol induced a reduction in renal functions revealed by increases in SCr and BUN. This deterioration might be attributed to decreasing GFR and renal blood flow secondary to renal vasoconstriction which eventually results in tubular damage, confirming that kidney is very sensitive to glycerol toxicity (Nath and Norby, 2000). However, pretreatment with MF (100mg/kg) for 10 days ameliorated AKI as manifested by the decrease in SCr, BUN, and improvements in histopathological features. Two studies reported that MF has a renoprotective effect against gentamicin-induced nephrotoxicity (Janjua et al., 2014) and renal ischemia/reperfusion injury of renal tubular epithelial cell (Wang et al., 2015).

Although the exact mechanisms by which rhabdomyolysis induces renal impairment are currently unclear, it is suggested that oxidative stress (Kim et al., 2010), inflammation (Homsi et al., 2009) and tubular apoptosis (Kim et al., 2010) all play a role.

Anti-peroxidatives like SOD and CAT enzymes protect the cells and tissues against oxidative stress-mediated injuries (Maheshwari et al., 2014). Results in the present study indicate that there was an increase in the oxidative stress with glycerol treatment revealed by the decrease in SOD and CAT activities. This might be due to the increased production of ROS which may cause inactivation and exhaustion of antioxidant enzymes such as SOD (Chander et al., 2003). It has been further shown that the activity of both SOD and CAT were increased upon pre-treatment with MF. Previous research has indicated that MF possesses antioxidant potential (Ouslimani et al., 2005). Furthermore, Maheshawari et al., (2014) reported a protective effect of MF against streptozotocin-nicotinamide-induced diabetic nephropathy mediated by increasing SOD and CAT activities.

IL-6 has been reported to increase the cell-mediated immune response that promotes renal injury (Nechemia-Arbely et al., 2008). According to our observations, glycerol increased the pro-inflammatory IL-6, while pre-treatment with MF declined IL-6 which may contribute to its anti-inflammatory effect against rhabdomyolysis-induced AKI. MF was showed to prevent endotoxin-induced liver injury, blunting the effect of endotoxins on the pro-inflammatory cytokine IL-6 (Nechemia-Arbely et al., 2008).

In rhabdomyolysis, lipid peroxidation induces distortion of mitochondrial membrane permeability, resulting in a drop in cellular respiratory control, with amplification of ROS production, cytochrome C release, activation of caspases 1 and 3,

and tubular-cell apoptosis (Panizo et al., 2015). Inhibition of caspase 1 and 3 resulted in amelioration of rhabdomyolysis-induced AKI (Homsi et al., 2006). That was consistent with our findings that MF-attenuated kidney injury was accompanied by declining active caspase-3 content which was conspicuously increased by glycerol treatment.

Further evidence for glycerol-induced acute renal failure was clearly observed in our histopathological studies. Severe degenerative changes in tubules, diffused cellular infiltration all over the parenchyma and cystic tubules with eosinophillic casts in the lumen of the tubules at the corticomedullary portion were observed in glycerol treated group. Damaged tubules and glomeruli appeared to be restored and only mild inflammation was noticed in MF/glycerol group. From these histopathological observations it is clear that MF is beneficial in restoring the glycerol-induced tissue damages in rats' kidney. Meanwhile, in accordance with our results, prevention of histologic changes due to gentamicin toxicity by MF was shown by Morales et al., (2010)

To the best of our knowledge, this is the first study to reveal a protective effect of MF against rhabdomyolysis-induced AKI. In conclusion, MF has the potential to be used in the prevention of rhabdomyolysis-associated AKI. MF-protective effect is, at least in part, due to its ability to balance oxidative stress, regulate the inflammatory and apoptotic mediators.

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الملخص العربى

التأثير الوقائى للميتفورمين ضد القصور الكلوي الحاد المحدث بالجليسرول في الجرذان

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الخلفية:

يعتبر الاعتلال الكلوي الحاد الناجم عن إنحلال الربيدات من إحدى الحالات الخطيرة التي تهدد الحياة لذلك فإنه من الضروري إيجاد طرق أكثر فعالية للحد من هذا المرض. تتضمن آليات هذا المرض طرق عدة منها الأكسدة والالتهاب وموت الخلايا المبرمج.

يعتبر الميتفورمين عقار ذو خواص مضادة لكل من الأكسدة والالتهاب والموت المبرمج للخلايا ،و قد أوضحت هذه الدراسة التأثير الوقائي المحتمل لمادة الميتفورمين على تطور الاعتلال الكلوي الحاد المحدث بمادة الجلسرول فى الجرذان.

الهدف:

كان الهدف من هذه الدراسة هو الكشف عن التأثير الوقائي المحتمل لمادة الميتفورمين على نموذج من الجرذان المصابة بالاعتلال الكلوي الحاد المحدث بواسطة الجلسرول حتى يمكننا الإمداد بأساسيات تجريبية لاستخدام الميتفورمين في الوقاية الإكلينيكية من الاعتلال الكلوي الحاد الناجم عن إنحلال الربيدات.

تم تقييم التأثير الوقائى، ومكافحة كلا من الأكسدة و الالتهاب وموت الخلايا المبرمج على نموذج الجرذان.

المواد والطرق المستخدمة:

لقد قمنا باستخدام أربعين جرذا فى هذه الدراسة تم تقسيمهم عشوائيا إلى أربع مجموعات متساوية:

(١): المجموعة الضابطة.

(٢):مجموعة تم إعطاؤها جلسرول ١٠مل/ كجم بالعضل.

(٣): مجموعة تم إعطاؤها جلسرول ١٠مل/ كجم بالعضل بعد ١٠ أيام من إعطاء الميتفورمين ١٠٠مجم/كجم بالفم.

(٤): مجموعة تم إعطاؤها الميتفورمين ١٠٠مجم/كجم بالفم.

تم فحص سلامة الكلى عن طريق اختبار وظائف الكلى بقياس الكرياتينين واليوريا فى الدم واختبارمعدل الأكسدة بقياس السوبر اكسيد ديسميوتاز والكاتالاز.

كما تم فحص معدل الالتهاب بقياس انترلوكين ٦و معدل موت الخلايا المبرمج بقياس محتوى إنزيم كاسباس ٣ و كذلك تم فحص النسيج الكلوي.

النتائج:

أدى إعطاء الجلسرول إلى:

زيادة في متوسط تلف النسيج الكلوي.

زيادة مستوى كل من الكرياتينين واليوريا فى الدم.

زيادة محتوى الإنترلوكين ٦ والكسباس ٣.

كما أدى إلى نقص نشاط كل من السوبر اكسيد ديسميوتاز والكاتالاز.

إلا أن كل هذه العوامل قد تحسنت بشكل كبير بإعطاء عقار الميتفورمين قبل الجلسرول.

الملخص والإستنتاجات:

قد إستنتجنا مما سبق أن العلاج المسبق بعقار الميتفورمين قد أدى إلى تحسن وظائف الكلى المتدهورة فى نموذج الاعتلال الكلوى الناتج عن إنحلال الربيدات والمحدث بالجلسرول وذلك عن طريق تثبيط كلا من الأكسدة والالتهاب وموت الخلايا المبرمج.