CHAPTER-3
TO INVESTIGATE THE ROLE OF NITRIC OXIDE
IN GLYCEROL-INDUCED MYOGLOBINURIC
ACUTE RENAL FAILURE
INTRODUCTION
Muscle accounts for approximately 40% of total body mass and it falls victim to a wide variety of toxic, ischemic, infectious, inflammatory, and metabolic insults. The final result of these diverse assaults may be muscle fiber dissolution or rhabdomyolysis resulting in the release of potentially toxic intracellular components into the systemic circulation.

Rhabdomyolysis is a common condition in which injury to the skeletal muscle results in leakage of the contents of myocytes into extracellular fluid and, if severe enough, passage of sufficient myoglobin into urine to colour it brown (Beetham, 2000). The rhabdomyolysis syndrome has been recognized for centuries. However, it was not until Bywaters’ and Beall’s classic description of the “crush syndrome”, a result of the bombing raids of London during World War II that the renal complications of rhabdomyolysis became firmly entrenched in the medical literature (Bywaters and Beall, 1941). Since then, a plethora of non-traumatic causes of muscle necrosis have been recognized and today rhabdomyolysis is considered a leading cause of ARF. In recent years, a number of important pathophysiological insights involved in rhabdomyolysis-induced ARF have emerged, offering a scope for development of potentially new prophylactic and therapeutic approaches.

MAIN CAUSES OF RHABDOMYOLYSIS
Direct muscle trauma
- Crush syndrome including immobility-induced compression
- Electrocution, burns

Ischaemia
- Vascular occlusion
- Compression

Excess Muscular activity
- Seizures
- Sports / Military training
- Body-building
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Drugs

- Alcohol, opiates, cocaine, tricyclics, amphetamines, neuroleptics, statins

Infection

- Bacterial
- Viral

Toxins

- Snake venom, insect venom

Inflammatory myopathies

- Polymyositis
- Dermatomyositis

Metabolic disorders

- Diabetic ketoacidosis
- Hypokalaemia
- Hypophosphataemia

Hereditary Disorders

- Carbohydrate metabolism, e.g., McArdle’s syndrome
- Lipid metabolism, e.g., carnitine palmitoyltransferase deficiency

Temperature Extremes

- Hyper / hypothermia

MUSCLE INJURIES WHICH CAN CULMINATE IN RHABDOMYOLYSIS

Crush Syndrome

Crush injuries sustained during the Battle of Britain formed the basis for Bywaters’ and Beall’s classic description of rhabdomyolysis-associated ARF (Bywaters and Bealls, 1941). While this form of injury is now seen most often as a sporadic event, it can still occur in “epidemic” proportions as tragically demonstrated by U.S. Marines bombing of Beriut barracks in 1983 and by the Armenian earthquake of 1988 in which 600 patients developed myoglobinuric ARF (Better, 1990). The pathophysiology of crush syndrome-induced muscle injury while seemingly straightforward is actually quite complex. The earliest
insult may be “pressure-stress myopathy” in which external pressure/tension on muscle caused increased sarcolemmal Na⁺ and Ca²⁺ influx down their concentration gradients (Christensen, 1987; Better, 1990). External pressure-induced occlusion of the microcirculation also occurs, rapidly depleting ambient and myoglobin oxygen content. While some high-energy phosphate reserves exist in the form of creatine phosphate, these stores are soon depleted, glycogen stores are exhausted and severe ATP depletion ensues. Despite this onerous situation, cell viability may remain intact for considerable periods of time, for a number of reasons (Odeh, 1991): (1) pressure induced vascular occlusion limits Ca²⁺ delivery to ischemic tissues; hence, this may stave off critical muscle Ca²⁺ loading for stalling the onset of cell necrosis. (2) Mitochondrial oxygen free radical production is markedly reduced during ischemia because oxygen deprivation stops electron transport; thus, decreased rather than increased mitochondrial H₂O₂ and superoxide production may result (Freeman and Crapo, 1982). (3) Intracellular/extracellular acidosis, a correlate of tissue ischemia, has a remarkably potent and apparently generalized cytoprotective effect (Zager et al., 1993). With restitution of blood flow, each of these intrinsic homeostatic mechanisms are reversed: Ca²⁺ influx occurs, brisk mitochondria oxygen free radical formation may result, and tissue acidosis recedes. Furthermore, restoration of blood flow allows for neutrophil influx, which can re-occlude the microenvironment, myoglobin gains ready access the circulation resulting in a new wave of injury (Odeh, 1991). These considerations indicate that although the crush lesion clearly sets the stage for rhabdomyolysis, many of its critical components are enacted during the reperfusion period.

**Exertional Rhabdomyolysis**

Sternuous physical exercise is probably the most common cause of severe rhabdomyolysis, particularly when it is performed by untrained individuals in hot, humid weather. Therefore, it is not surprising that outbreak of myonecrosis has been noted at southern military based and summer sports training camps (Knochel, 1990). However, non-athletic exertion can also
produce this result, as evidenced by the onset of myoglobinuria following generalized convulsions or septic rigors. The specific events leading to exertional myonecrosis have only been partially clarified, and the syndrome develops in stages. The initiating stage is believed to result from mechanical muscle fiber injury induced in particular by eccentric contractions and when the muscle is in an elongated condition such as down hill walking (Armstrong et al., 1991; Kuipers, 1994). These factors produce excessive tensile stress and heat generation resulting in muscle capillary blood flow. When coupled with progressive glycogen and creatine high-energy phosphate consumption, ATP depletion results. The Ca\(^{2+}\) overload stage is thought to be initiated by a Ca\(^{2+}\) influx across a mechanically damaged myocyte sarcolemmal membrane (Lopez et al., 1995). Ca\(^{2+}\) overload is then believed to initiate the autogenous phase, during which phospholipase and protease activation, mitochondrial dysfunction, and free radical formation perpetuate sarcolemmal injury, culminating in cell death. Muscle hyperthermia (as much as 43°C), induced by strenuous exercise in concert with impaired heat dissipation (by hot humid conditions), undoubtedly plays a major role in these processes, for at least two reasons. First, rising muscle temperature markedly increases metabolic rate, and hence, ATP consumption. While this may be well tolerated by otherwise normal tissues, it can cause profound ATP depletion in cells with already compromised glycolytic or mitochondrial energy production (Zager et al., 1991). Second, enzymatic degradative reactions are highly thermal dependent, generally increasing by \(~10\%\) for each 1°C increment. This not only accelerates tissues injury, but it can also convert sublethal into lethal cell damage (Zager and Altschuld, 1986).

Of considerable interest is the mechanism by which prior muscle conditioning prevents the above sequence of events (Knochel, 1990). It presumably reflects a cellular adaptation to prior exercise induced sublethal injury. The exact pathways by which it is affected remain to be defined.
Intrinsic Myopathies

The first report of a presumptive metabolic disorder causing recurrent myonecrosis appeared in 1884 (Frohner, 1884). Approximately 30 minutes after the onset of strenuous exercise, horses were noted to develop muscle tremors, weakness and limpness followed by myoglobinuria. The study of hereditary disorders that trigger rhabdomyolysis began in 1910. Since that time, a panoply of muscle energy production disturbances have been documented, of which McArdle’s disease is a prototype.

McArdle’s disease is the most common cause of the first set of disorders and is believed to be due to myophosphorylase deficiency. Since this enzyme promotes glycogenolysis, its deficiency presumably causes defective anaerobic energy production in type II fibers, culminating in exercise-induced ATP depletion and myonecrosis (Dmaison and Grynberg, 1994).

Malignant Hyperthermia

Another muscle disorder leading to rhabdomyolysis is represented by malignant hyperthermia. Classically, it presents with the onset of muscle rigidity and contractures, a rapidly rising body temperature (as much as 1°C every 5 min; reaching ≥ 43°C) and myonecrosis. Although the exact genetic and metabolic basis for this disorder remains unknown, it is believed to be due to altered excitation-contraction coupling, resulting in increased sarcolemmal Ca\(^{2+}\) transport, contractures and increased muscle heat production (Brumback et al., 1992).

Alcoholism, hypophosphatemia and hypokalemia

Alcoholism is a leading cause of rhabdomyolysis (Gabow et al., 1982) and may contribute to muscle injury in a variety of ways. First it is a direct muscle toxin, inducing either chronic or acute myopathy. It also induces substantial changes in muscle ion homeostasis, malnutrition, including increments in Na\(^+\), Cl\(^-\), and Ca\(^{2+}\), and decrements in Mg\(^{2+}\), K\(^+\), and phosphate content. These changes are associated with increased Na\(^+\), K\(^+\)-ATPase activity, suggesting that ethanol directly injures the sarcolemma, allowing for increased Na\(^+\) entry (Knochel, 1993). A secondary increase in Ca\(^{2+}\) may then result via enhanced
Na\(^{2+}\)/Ca\(^{2+}\) exchange. Due to calcium's critical role in mediating rhabdomyolysis, these processes may either initiate myonecrosis or predispose the muscle to concomitant insults, such as electrolyte disturbances.

Hypophosphatemia is a frequent co-existent risk factor for alcohol-induced rhabdomyolysis (Knochel, 1993), although the direct mechanisms by which it does so have remained somewhat elusive. A leading possibility is that intracellular inorganic phosphate deficiency limits ATP production, potentially imposing an energy deficient state. The latter would then predispose to intracellular Ca\(^{2+}\) loading, ultimately triggering myocyte death. Hence, it is postulated that phosphate depletion only initiates myonecrosis in the presence of pre-existent muscle damage. This is particularly likely to occur during a period of caloric, but phosphate deficient, refeeding (such as, glucose infusion). A possible reason for this is that cellular glucose uptake obligates phosphate utilization (glucose 6-phosphate formation), potentially triggering severe intracellular inorganic phosphate and ATP-depletion. With the onset of muscle necrosis, intracellular phosphate is released. This may rapidly correct the hypophosphatemia, thereby, obscuring its original pathogenetic contribution to the muscle injury.

Hypokalemia is another electrolyte disturbance, which may initiate or contribute to rhabdomyolysis-induced ARF (Knochel, 1993). Common underlying causes include physical exercise in hot weather (sweat and urinary K\(^{+}\) losses), long acting diuretic therapy and alcoholism. As with acute hypophosphatemic myopathy, it may be difficult to document hypokalemia with the onset of myonecrosis because of the resulting cellular K\(^{+}\) loss. Two mechanisms for hypokalemic rhabdomyolysis have been suggested, both of which relate to altered myocyte energetics. First K\(^{+}\) depletion can interfere with glycogen synthesis. Second, the exercising muscle may fail to develop reactive hyperemia, a prerequisite for increased oxygen delivery during a period of increased oxygen demand (Knochel, 1990). Since this vasodilatory response is dependent on myocyte K\(^{+}\) release and the attainment of high muscle interstitial K\(^{+}\) concentrations (~10 to 15 m Eq/liter), a pre-existent K\(^{+}\)
deficiency may prevent this critical adaptive response. Thus a combination of diminished glycogen reserves and impaired oxygen delivery/aerobic energy production may initiate myocyte death (Knochel, 1990).

**MECHANISMS OF MYOHEMOGLOBINURIC RENAL INJURY**

The association of rhabdomyolysis with ARF was well established at the time of World War I. However, direct involvement of myoglobin was not definitively established until Bywaters and Stead demonstrated induction of ARF by myoglobin infusion (Bywaters and Stead, 1944). These experimental studies also revealed the two critical factors which predispose to myohemoglobinuric ARF: hypovolemia/dehydration and aciduria. Indeed, it has been repeatedly demonstrated that in their absence, heme proteins have minimal nephrotoxic effects. At the nephronal level, three basic mechanisms underlie heme protein toxicity: (I) renal vasoconstriction, (II) intraluminal cast formation, and (III) direct heme protein–induced cytotoxicity (Zager, 1992a)

**Renal Vasoconstriction:**

The two most widely used experimental models of heme protein-induced ARF are either heme protein (hemoglobin/myoglobin) infusion into dehydrated/volume depleted animals or intramuscular hypertonic glycerol injection. The latter causes myolysis, hemolysis and intravascular volume depletion. The importance of the latter and its associated renal vasoconstriction are underscored by the fact that either volume expansion or early renal vasodilator therapy can prevent, or profoundly attenuate, heme protein-induced ARF. Intravascular volume depletion is a near constant correlate of clinical rhabdomyolysis, and that hemoglobinuria rarely induces ARF in volume-expanded individuals (Zager, 1994), indicating the relevance of these experimental observations.

Several potential mechanisms may contribute to renal vasoconstriction/hypoperfusion in the setting of rhabdomyolysis: First, muscle necrosis caused dramatic fluid third spacing, leading to intravascular volume depletion. Better et al (1990) indicated that following severe crush injuries, as much as 18 liters fluid may extravasate into damaged limb. An aggressive volume repletion
(~1 liter/hr) during the early post-injury period can dramatically decrease the incidence of clinical rhabdomyolysis-induced ARF. Second, severe muscle injury can, for uncertain reasons, activate the endotoxin-cytokine cascade (Shulman et al., 1993; Vogt et al., 1995). Third, heme proteins scavenge nitric oxide (NO), an important endogenous vasodilator (Furchgot and Jothianandan, 1991). Studies by Brezis et al. (1991) have demonstrated importance of NO in maintaining renal medullary oxygenation, an area with a tenuous blood supply. Interventions, which increase and decrease NO protect against and exacerbate various forms of experimental ARF, respectively, underscoring the potential importance of NO in the expression of toxic and ischemic tubular damage (Agmon et al., 1994; Amore et al., 1995). Finally, Wright, Rees and Moncada have indicated that NO synthase inhibition in experimental septic shock markedly increases vasoconstriction, producing organ tissue damage and rapid death (Wright et al., 1992). Hence, it seems clear that heme protein-induced NO scavenging could directly contribute to renal hypoperfusion and tissue injury in the setting of rhabdomyolysis.

Above studies strongly suggest that renal vasoconstriction participates in the expression of heme protein-associated ARF via the induction of renal tubular ATP depletion. However, renal vasoconstriction can also facilitate heme toxicity by increasing cast formation and by promoting proximal tubular cell heme uptake. This occurs via a number of pathways; first, a decrease in GFR prolongs the circulating half-life of heme proteins. Since heme endocytosis is a saturable process, most of the filtered load is rapidly excreted. However if the period of heme proteinemia is prolonged, due to decrease in GFR, more of the total filtered load can undergo reabsorption, thereby exacerbating the extent of proximal tubular heme loading, and hence, cell damages. Second, volume depletion, and hence increased sodium and fluid reabsorption, increase intraluminal myoglobin concentrations, favoring cast formation and tubular obstruction; and third, tubular obstruction causes profound luminal stasis, allowing more time for proximal tubular heme reabsorption (Zager and Gamelin, 1989). These considerations merely underscore that renal
vasoconstriction can adversely impact the expression of myohemoglobinuric injury via divergent, but interrelated pathways.

**Heme protein cast formation**
The presence of large numbers of distal nephron heme pigment casts has been noted in virtually every study of myohemoglobinuric ARF (Baker and Dodds, 1925; Jaenike, 1967). It has been widely hypothesized that these casts induce tubular obstruction, thereby contributing to the pathogenesis of the renal failure. Despite this assumption, consistent micropuncture data to support this view have been lacking.

The mechanism by which heme protein cast formation occurs has been rather well characterized and is largely determined by two factors: (1) the heme protein concentration in the distal nephron; and (2) the characteristics of the urine in which it resides. Since the degree to which heme proteins precipitate from filtered load (determined in large part by the extent of muscle injury), and more that load is concentrated along the nephron, the more likely it is that cast formation will result. Since volume depletion and renal vasoconstriction enhance filtrate reabsorption, they can increase intraluminal heme concentrations, facilitating cast development.

**Direct heme protein-induced cytotoxicity:**
Heme proteins exert direct cytotoxic effects on proximal tubules via two critical factors: ischaemic damage and oxidant tissue stress.

**Ischemic tubular injury and ATP depletion**
It has been recognized for at least 50 years that heme protein nephrotoxicity and ischemic renal damage are inextricably linked (Lowe, 1966). This is probably mediated in part by the ability of heme proteins to intensify renal vasoconstriction, thereby converting an episode of renal hypoperfusion into a severe ischemic insult. If an ordinarily non-toxic dose of hemoglobin or myoglobin is infused into a rat prior to the induction of a fixed ischemic renal insult (renal artery occlusion; RAO), a marked potentiation of ischemic tubular necrosis and filtration failure results. Another pathway through which heme proteins can contribute to ischemic tubular damage is via an apparent
adverse effect on cellular energetics, independent of their negative impact on renal perfusion.

Figure 1: Pathogenesis of rhabdomyolysis-induced nephrotoxicity

When normal rats were subjected an intravenous myoglobin infusion, an approximate 25% reduction in renal cortical ATP concentrations developed without any discernible change in renal hemodynamics (Zager, 1991). This result could not be reproduced by infusing a non-iron containing low molecular weight protein (ribonuclease), suggesting an iron dependent reaction. This conclusion was further supported by the fact that when myoglobin was infused in the presence of an iron chelator, desferrioxamine, no diminution in ATP resulted. In summary, these considerations underscore that multiple and potentially overlapping mechanisms for the heme protein-
induced exacerbation of ischemic renal injury exist: (i) heme proteins can intensify renal vasoconstriction in the setting of volume depletion; (ii) they can adversely affect ATP availability via a non hemodynamic, iron dependent mechanism; and (iii) heme protein endocytic reabsorption can directly sensitize tubular cells to the adverse consequences of superimposed ischemia-triggered injury pathways (such as PLA2 attack).

**Heme protein-induced oxidant stress**

In 1969, Bunn and Jandi injected haptoglobin deficient animals with tracer amounts of 59Fe labeled hemoglobin to determine its metabolic handling by the kidney. They noted that the hemoglobin rapidly dissociated within the circulation to αβ dimers (therapy halving its size, permitting rapid filtration) and this was followed by proximal tubule heme endocytic reabsorption. Once within proximal tubular cells, the porphyrin ring was rapidly catabolized, releasing its iron content. The latter was then transferred to ferritin. Subsequently, the intratubular 59Fe disappeared over several weeks. In contrast, if large amounts of hemoglobin were administered, the tubular reabsorptive capacity was exceeded, producing marked hemoglobinuria and intraluminal free iron release (Zager, 1992b). Iron-induced oxidant stress has become a well-accepted mediator of tissue damage (Johnson et al., 1986).

Taken together it seems highly plausible that "catalytic" iron (that is, capable of promoting free radical reactions) could play a critical role in heme-induced nephrotoxicity. Nitric Oxide (NO) scavenging induced by heme proteins could directly contribute to renal hypoperfusion and tissue injury in the setting of rhabdomyolysis.

Thus in the present study, various pharmacological agents targeted to release or increase the nitric oxide levels by nitric oxide donation or by the agents upregulating the nitric oxide synthase (NOS) enzyme, were evaluated for their effect on glycerol induced myoglobinuric ARF in rats.
MATERIALS AND METHODS
Male wistar rats (150-200g), bred in the central animal house of Panjab University (Chandigarh, India) were used. The animals were housed under standard conditions of light and dark cycle with free access to food and tap water. All the protocols were approved by the Institutional Animal Ethics Committee of the Panjab University.

Drugs
Glycerol (Ranbaxy Laboratories, Mohali, India), was dissolved in normal saline to give a final strength of 50% v/v. L-arginine (Himedia, Mumbai), Pravastatin (Ranbaxy Research Laboratories, Gurgaon, India), L-NAME (Sigma, USA), aminoguanidine (Himedia, Mumbai), L-NIO (Caymen Chemicals, USA) were dissolved in distilled water. Nebivolol (Jenessan Pharmaceutica, Belgium), resveratrol (Sigma, St. Louis, MI, USA), molsidomine (Caymen Chemicals, USA) were suspended in 0.25% carboxy methyl cellulose (CMC). All the drugs were freshly prepared at the beginning of each experimental protocol.

EXPERIMENTAL PROTOCOL
Rats were allowed free access to food but deprived of water for 24 hours before the glycerol injection. After dehydration the animals were injected with 50% hypertonic glycerol (8ml/kg) as a divided dose into the hind limbs. All the animals were kept in individual cages, for the first few hours, with free access to food and tap water to recover from any initial muscle injury after intramuscular injection. Then the animals were placed in individual metabolic cages to measure water intake and urine output. The animals were sacrificed with a high dose of anaesthesia 24 hours after the glycerol injection and blood was collected in centrifuge tubes through abdominal aorta. Serum was isolated and was used freshly for the assessment of renal function tests. A midline abdominal incision was performed and both the kidneys were isolated, the left kidney was deep frozen till further enzymatic analysis, whereas, the right kidney was stored in 10% formalin for the histological sectioning.

Assessment of renal function
Serum samples were assayed for blood urea nitrogen (BUN) and serum creatinine by using standard diagnostic kits (Span Diagnostics, Gujarat, India).
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Estimation of tissue and urine nitrite and nitrate levels:
As per chapter 1.

Post mitochondrial supernatant preparation (PMS)
As per chapter 1.

Estimation of lipid peroxidation
As per chapter 1.

Estimation of antioxidant enzymes (AOE)
As per chapter 1.

Renal Histology
Kidneys were processed in the same way as described in chapter 1, but the renal sections were examined in blind fashion for hemorrhagic and hyaline casts, tubular necrosis and apical blebbing in all treatments.

STATISTICAL ANALYSIS
Data are presented as means ± S.E.M. One way analysis of variance (ANOVA) followed by Student-Newman-Keuls test was applied to calculate the statistical significance between various groups. A value of $p<0.05$ was considered to be statistically significant.

STUDY DESIGN
Animals were divided into twenty one groups, each comprising of eight animals. The animals were allowed free access to food but deprived of drinking water for 24hr before glycerol injection.

Group I (C) comprised of animals that received an equivalent volume of saline for glycerol.

Group II (G) animals received an intramuscular injection of 8ml/kg hypertonic glycerol as a divided dose into the hind limbs.

Groups administered NO precursor (L-arginine) and NOS inhibitors

Group III (L-arg+G) animals were given L-arginine (125 mg/kg, i.p.), 30 minutes prior to the glycerol injection.
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**Group IV (L-NAME+L-arg+G)** animals were given L-NAME (10 mg/kg, i.p.), 60 minutes and L-arginine (125 mg/kg, i.p.), 30 minutes prior to the glycerol injection.

**Group V (L-NIO+L-arg+G)** animals were given L-NIO (5 mg/kg, i.p.), 60 minutes and L-arginine (125 mg/kg, i.p.), 30 minutes prior to the glycerol injection.

**Group VI (Amg+L-arg+G)** animals were given aminoguanidine (100 mg/kg, i.p.), 60 minutes and L-arginine (125 mg/kg, i.p.), 30 minutes prior to the glycerol injection.

Groups administered NO donors (molsidomine, nebivolol) and NOS inhibitors

**Group VII (Mol+G)** animals were given molsidomine (10 mg/kg, p.o.), 30 minutes prior to the glycerol injection.

**Group VIII (L-NAME+Mol+G)** animals were given L-NAME (10 mg/kg, i.p.), 60 minutes and molsidomine (10 mg/kg, p.o.), 30 minutes prior to the glycerol injection.

**Group IX (Neb+G)** animals were given nebivolol (1 mg/kg, p.o.), 30 minutes prior to the glycerol injection.

**Group X (L-NAME+Neb+G)** animals were given L-NAME (10 mg/kg, i.p.), 60 minutes and nebivolol (1 mg/kg, p.o.), 30 minutes prior to the glycerol injection.

Groups administered NOS upregulators (resveratrol, pravastatin) and NOS inhibitors

**Group XI (Rvt+G)** animals were given resveratrol (5 mg/kg, p.o.), 30 minutes prior to the glycerol injection.

**Group XII (L-NAME+Rvt+G)** animals were given L-NAME (10 mg/kg, i.p.), 60 minutes and resveratrol (5 mg/kg, p.o.), 30 minutes prior to the glycerol injection.

**Group XIII (L-NIO+Rvt+G)** animals were given L-NIO (5 mg/kg, i.p.), 60 minutes and resveratrol (5 mg/kg, p.o.), 30 minutes prior to the glycerol injection.

**Group XIV (Amg+Rvt+G)** animals were given aminoguanidine (100 mg/kg, i.p.), 60 minutes and resveratrol (5 mg/kg, p.o.), 30 minutes prior to the glycerol injection.

**Group XV (Pra+G)** animals were given pravastatin (20 mg/kg, i.p.), 30 minutes prior to the glycerol injection.

**Group XVI (L-NAME+Pra+G)** animals were given L-NAME (10 mg/kg, i.p.), 60 minutes and pravastatin (20 mg/kg, i.p.), 30 minutes prior to the glycerol injection.
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Group XVII (L-NIO+Pra+G) animals were given L-NIO (5 mg/kg, i.p.), 60 minutes and pravastatin (20 mg/kg, i.p.), 30 minutes prior to the glycerol injection.

Group XVIII (Amg+Pra+G) animals were given aminoguanidine (100 mg/kg, i.p.), 60 minutes and pravastatin (20 mg/kg, i.p.), 30 minutes prior to the glycerol injection.

Groups administered NOS inhibitors

Group XIX (L-NAME+G) animals were given L-NAME (10 mg/kg, i.p.), 30 minutes prior to the glycerol injection.

Group XX (L-NIO+G) animals were given L-NIO (5 mg/kg, i.p.), 30 minutes prior to the glycerol injection.

Group XXI (Amg+G) animals were given aminoguanidine (100 mg/kg, i.p.), 30 minutes prior to the glycerol injection.

RESULTS

Effect of L-arginine, molsidomine, nebivolol, resveratrol, pravastatin, L-NAME, L-NIO and aminoguanidine on glycerol-induced renal dysfunction

Glycerol (8 ml/kg, i.m.) administration resulted in a significant increase in serum creatinine (3.77 ± 0.31 mg/dl V/s 1.03 ± 0.025 mg/dl), and blood urea nitrogen (BUN) (28.63 ± 0.8 mg/dl V/s 11.62 ± 1.9 mg/dl) levels and decreased the creatinine and urea (0.099 ± 0.006 ml/min V/s 0.46 ± 0.01 ml/min & 0.03 ± 0.008 ml/min V/s 0.44 ± 0.023 ml/min) suggesting a significant degree of glomerular dysfunction.

Pretreatment of rats with L-arginine (125 mg/kg, i.p.), molsidomine (10 mg/mg, p.o.), nebivolol (1 mg/kg, p.o.), resveratrol (5 mg/kg, p.o.) and pravastatin (20 mg/kg, i.p.) produced a significant reduction in serum creatinine and urea nitrogen levels and a significant increase in creatinine and urea clearance associated with intramuscular glycerol injection. Prior treatment of animals with L-NAME/L-NIO significantly abolished the protective effect of L-arginine, resveratrol and pravastatin, while, aminoguanidine did not had any effect on the protective effect of these agents. However, L-NAME could not abolish the protective effect of molsidomine and nebivolol on renal dysfunction induced by glycerol administration. Treatment of animals with L-NAME/L-NIO further worsened the renal injury observed with glycerol (Fig.2a.-d., 3a-d.).
Effect of L-arginine, molsidomine, nebivolol, resveratrol, pravastatin, L-NAME, L-NIO and aminoguanidine on glycerol-induced urine and tissue total NO levels

Glycerol administration significantly reduced the urine (6.98±0.31 V/s 25.45±0.96 μmole/L) as well as tissue (2.01±0.09 V/s 12.18±0.4 μmole/mg protein) total nitric oxide levels. Treatment of animals with L-arginine, molsidomine, nebivolol, resveratrol and pravastatin exhibited a significant increase in urine as well as tissue total NO levels. Prior treatment of L-NAME/L-NIO blocked the protective effect of L-arginine, resveratrol and pravastatin on urine and tissue total NO levels in glycerol treated animals, while aminoguanidine failed to do so. However, L-NAME could not block the protective effect of molsidomine and nebivolol in urine and tissue total NO levels in glycerol administered animals. The treatment of animals with L-NAME/L-NIO alone exhibited almost same NO levels in glycerol treated animals (Fig.2e,2f. and Fig.3e,3f.).

Effect of L-arginine, molsidomine, nebivolol, resveratrol, pravastatin, L-NAME, L-NIO and aminoguanidine on glycerol-induced changes in TBARS and renal antioxidant pool

Treatment with glycerol significantly increased the TBAR levels, while the enzymatic activities of reduced glutathione, superoxide dismutase and catalase were reduced significantly. This increase in TBARS as well as reduction in renal antioxidative enzymes was significantly improved by treatment with L-arginine, molsidomine, nebivolol, resveratrol and pravastatin. Prior treatment of animals with L-NAME/L-NIO abolished the protective effect of L-arginine, resveratrol and pravastatin on increase in TBARS and reduction in antioxidative enzymes induced by glycerol, however prior treatment of animals with L-NAME could not abolish the protective effect of molsidomine and nebivolol in these parameters. Aminoguanidine treatment was not able to revert the protective effect of any of these agents in glycerol treated animals. The animals treated with L-NAME/L-NIO along with glycerol exhibited similar results as that of glycerol treatment (Fig.2g-j., Fig.3g-j.).

Effect of L-arginine, molsidomine, nebivolol, resveratrol, pravastatin, L-NAME, L-NIO and aminoguanidine on glycerol-induced changes in the renal morphology

The histopathological changes were graded and summarized in table 1. The control group did not show any morphological changes. By contrast, the kidneys of rats treated with
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glycerol showed marked histological changes in the cortex and outer medulla. The renal sections showed severe epical blebbing, hyaline casts, tubular necrosis and haemorrhagic casts. Treatment with L-arginine (125 mg/kg, i.p.), molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, i.p.) preserved the normal morphology of the kidney and showed normal glomeruli except for slight casts in the tubular cells. Prior treatment of animals with L-NAME/L-NIO reversed the morphological protection observed with L-arginine, resveratrol and pravastatin to a greater extent, while L-NAME treatment only partially reversed the morphological protection observed with molsidomine and nebivolol. The animals treated with L-NAME/L-NIO and glycerol showed similar morphology as that of glycerol treated animals. Administration of the same doses of the L-arginine, molsidomine, nebivolol, resveratrol and pravastatin to control animals did not result in any significant change in the renal function, antioxidant profile and renal morphology.
Effect of L-arginine (125 mg/kg, i.p.), resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on serum creatinine (Fig.2a.) and blood urea nitrogen (BUN) (Fig.2b.) in rats treated with glycerol. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to glycerol treated group, b p<0.05 as compared to L-arg + G, RVT + G, Pra + G group (one-way ANOVA followed by Newman Keuls test).
Effect of L-arginine (125 mg/kg, i.p.), resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on creatinine clearance (Fig. 2c.) and urea clearance (Fig. 2d.) in rats treated with glycerol. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a *p<0.05 as compared to glycerol treated group, b *p<0.05 as compared to L-arg + G, RVT + G, Pra + G group (one-way ANOVA followed by Newman Keuls test).
Effect of L-arginine (125 mg/kg, i.p.), resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg,i.p.), L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on urine (Fig.2e.) and tissue total NO levels (Fig.2f.) in rats treated with glycerol. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to glycerol treated group, b p<0.05 as compared to L-arg + G, RVT + G, Pra + G group (one-way ANOVA followed by Newman Keuls test).
Effect of L-arginine (125 mg/kg, i.p.), resveratrol (5 mg/kg, p.o.), pravastatin (20 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on TBARS (Fig.2g.) and reduced glutathione (Fig.2h.) in rats treated with glycerol. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to glycerol treated group, b p<0.05 as compared to L-arg + G, RVT + G, Pra + G group (one-way ANOVA followed by Newman Keuls test).
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Fig. 2j.

Effect of L-arginine (125 mg/kg., i.p.), resveratrol (5 mg/kg., p.o.), pravastatin (20 mg/kg., i.p.), L-NAME (10 mg/kg., i.p.), L-NIO (5 mg/kg., i.p.) and aminoguanidine (100 mg/kg., i.p.) on SOD (Fig. 2i.) and catalase (Fig. 2j.) in rats treated with glycerol. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to glycerol treated group, b p<0.05 as compared to L-arg + G, RVT + G, Pra + G group (one-way ANOVA followed by Newman Keuls test).
Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), L-NAME (10 i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on serum creatinine and blood urea nitrogen (BUN) in rats treated with glycerc.

Values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a as compared to glycerol treated group, b p<0.05 as compared to Mol + G or Nc (one-way ANOVA followed by Newman Keuls test).
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Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), L-NAME (10 mg/kg, i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on creatinine clearance (Fig.3c.) and urea clearance (Fig.3d.) in rats treated with glycerol. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to glycerol treated group, b p<0.05 as compared to Mol + G or Neb + G (one-way ANOVA followed by Newman Keuls test).
Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), L-NAME (10 n i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on urine (Fig.3e) tissue total NO levels (Fig.3f.) in rats treated with glycerol. The values are expressed as mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to gl; treated group, b p<0.05 as compared to Mol + G or Neb + G (one-way ANOVA followed by Newman Keuls test).
Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), L-NAME i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on TBAF and reduced glutathione (Fig.3h.) in rats treated with glycerol. The values are mean ± S.E.M. *p<0.05 as compared to control group, a p<0.05 as compared to glycerol treated group, b p<0.05 as compared to Mol + G or Neb + G (one-way followed by Newman Keuls test).
Effect of molsidomine (10 mg/kg, p.o.), nebivolol (1 mg/kg, p.o.), L-NAME (10 n i.p.), L-NIO (5 mg/kg, i.p.) and aminoguanidine (100 mg/kg, i.p.) on SOD (Fig.3i) and catalase (Fig.3j) in rats treated with glycerol. The values are expressed as mean ± S. *p<0.05 as compared to control group, a p<0.05 as compared to glycerol treated, **p<0.05 as compared to Mol + G or Neb + G (one-way ANOVA followed by Ne Keuls test).
Hematoxylin-Eosin stained longitudinal sections of kidneys of normal treated rat (B), L-arg+Gly treated rat (C), L-NAME+L-arg+Gly treated rat (D), NIO+L-arg+Gly treated rat (E) and Amg+L-arg+Gly treated rat (F).
Hematoxylin-Eosin stained longitudinal sections of kidneys of Mol+Gly treated (G), L-NAME+Mol+Gly treated rat (H), Neb+Gly treated rat (I), L-NAME+Neb+Gly treated rat (J), RVT+Gly treated rat (K) and L-NAME+RV treated rat (L) (312.5X)
Hematoxylin-Eosin stained longitudinal sections of kidneys of L-NICH treated rat (M), Amg+RVT+Gly treated rat (N), Pra+Gly treated rat (P), NAME+Pra+Gly treated rat (Q), L-NIO+Pra+Gly treated rat (R) (312.5X)
DISCUSSION

Since its description by Bywaters and Beall (1941), the relationship between rhabdomyolysis and acute renal failure has drawn considerable attention of nephrologists. Clinical experience in disastrous situations shows that predisposing intravascular volume depletion with or without metabolic acidosis appears to be essential for the development of ARF (Ron et al., 1984). However, a direct causal relationship between muscle injury and ARF has been suggested from an accumulation of cases with non-traumatic rhabdomyolysis, in which clinically apparent circulatory disturbance is minimal (Grossman et al., 1974).
The pathophysiology of rhabdomyolysis-induced myoglobinuric acute renal failure has been studied extensively in the animal model of glycerol-induced ARF (Nath et al., 1992; Zager, 1996; Baliga et al., 1996; Shimizu et al., 1998; Ferraz et al., 2002; Chander et al., 2003). The main pathophysiologic mechanisms are renal vasoconstriction, intraluminal casts and direct heme-protein induced cytotoxicity (Zager, 1996). Several potential mechanisms may contribute to renal vasoconstriction/hypoperfusion in the setting of rhabdomyolysis. First, muscle necrosis causes dramatic fluid third spacing, leading to intravascular volume depletion. This third spacing effect causes hypovolemia and hypotension, which impairs renal perfusion. Second, severe muscle injury can activate the endotoxin cytokine cascade that may lead to renal vasoconstriction finally. Third, heme proteins scavenge NO, a potent renal vasodilator, causing renal vasoconstriction and thus reducing renal blood flow (Maree et al., 1994; Zager, 1996; Valdivielso et al., 2000).

The intramuscular administration of hypertonic glycerol induces myolysis and hemolysis and is being widely utilized as a model of heme protein-induced renal injury (Ayer et al., 1971; Zager, 1996). The heme protein-induced renal injury represents the integrated effects of three major pathophysiologic mechanisms: renal vasoconstriction, direct cytotoxicity, and cast formation (Dubrow and Flamenbaum, 1988; Zager, 1996). The pigments (Hemoglobin and Myoglobin), themselves are unlikely to induce acute renal failure, but their presence within the systemic circulation during periods of acidosis, dehydration, shock, or other conditions associated with reduced renal perfusion may lead to both direct toxic and hemodynamic abnormalities resulting in acute renal failure (Dubrow and Flamenbaum, 1988).

In the present study, all the animals were dehydrated for 24 hours. Prior dehydration allows the full expression of renal injury, particularly the cast formation, compared to the non-dehydrated model (Zager, 1996). The animals lost an average of 5-8% of their body weight in the period of dehydration, during which time their food intake was one third less than that in the non-dehydrated state. Moreover, oliguria, or anuria developed only in the rats, which were dehydrated prior to the glycerol injection. The urinary volume of dehydrated control animals was one half of the urinary volume in non-dehydrated animals (data not shown). Furthermore, micropuncture studies have shown that the
glomerular filtration rate of dehydrated controls falls significantly as compared to non-dehydrated controls. Glycerol administration led to a significant decrease in urea and creatinine clearance, increase in serum creatinine, BUN levels, an increase in MDA levels, which reflects lipid peroxidation, a significant decrease in activities of GSH, CAT and SOD enzyme activities and severe renal morphological impairment. In addition, NO (urine as well as tissue total NO levels), which is an important mediator in the pathophysiology of this model, was also decreased with glycerol administration.

One of the most important and earliest events occurring after glycerol injection is a reduction of renal blood flow (RBF) (Ayer et al., 1971; Venkatachalan et al., 1976). It has been demonstrated that animals treated with glycerol demonstrate a decreased NO production. This decrease may play a role in the decreased RBF observed in these animals, as NO is a potent vasodilator (Valdivielso et al., 2000). After glycerol injection, myoglobin and hemoglobin are released into the circulation. These pigments contain a heme group, which shows strong affinity for NO. Thus, the binding of NO to heme group would lead to a decrease in NO activity and also a decrease in NO oxidation to nitrite. Nevertheless, this approach has also produced the view that heme-iron-driven hydroxyl radical (OH) generation is a critical mediator of the evolving tubular damage. This conclusion is supported by the following pieces of information: (1) iron chelation (Deferoxamine) therapy partially mitigates the extent of tubular necrosis and filtration failure (Paller, 1988; Chander et al., 2003); (2) OH scavengers (Shah and Walker, 1988; Zager, 1996) and glutathione (Abull-Ezz et al., 1991) can exert protective effects; (3) lipid peroxidation, a biochemical hallmark of oxidative stress, has been reported in the aftermath of heme protein nephrotoxicity (Paller, 1988); and (4) induction or suppression of heme oxygenase (HO* the enzyme which degrades heme porphyrin), has been shown to decrease or increase the severity of myohemoglobinuric (glycerol) ARF, respectively (Nath et al., 1992).

Pretreatment of animals with L-arginine, resveratrol and pravastatin markedly reduced the renal dysfunction, significantly improved the urine as well as tissue total NO levels, significantly improved the levels of renal oxidative enzymes and improved the renal morphological architecture. However, the protection afforded by these agents was significantly attenuated by prior treatment with L-NAME, suggesting that these agents
exerted their protective effect through release of NO. Since these effects were also abolished by prior treatment with L-NIO, a specific eNOS inhibitor, it is clear that these agents exert their effect through upregulation of eNOS, thereby increasing the NO levels, however same was not the case with aminoguanidine, a specific iNOS inhibitor. In case of animals treated with RVT, the difference was significant from animals treated with L-NAME as well as L-NIO in almost all the parameters observed, except the urine and tissue NO levels. The reason for this might be that RVT has also been shown to possess strong antioxidative activity.

Pretreatment of glycerol treated animals with molsidomine and nebivolol prevented the alterations produced by glycerol administration. However, prior treatment of animals with L-NAME was not able to abolish the protective effect of these two agents indicating that these two agents exerted their protective effect through their NO donating property, rather that altering the NOS enzyme.

The present results seem to support the pathogenic importance of the nitric oxide in glycerol-induced acute renal failure. Our results suggest the use of agents which can either donate NO or which can upregulate the eNOS enzyme, may have potential in treatment of rhabdomyolysis-induced myohemoglobinuric acute renal failure.