The Possible Protective and Therapeutic Effect of Apocynin on the Rhabdomyolysis-Induced Acute Kidney Injury in Albino Rats

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Abstract

Background: Rhabdomyolysis-induced Acute Kidney Injury (AKI), develops following skeletal muscle trauma, is associated with high morbidity and mortality. The pathophysiology of AKI in rhabdomyolysis is likely complex and incompletely understood.

Aim: This study aimed to investigate the protective and the therapeutic effect of apocynin on rhabdomyolysis-induced AKI in albino rats.

Methods: Rats were divided as following: Group 1 serves as the control, Group 2 was given 50% glycerol, Group 3 was given glycerol after 7 days pretreatment with apocynin, and group 4 was given a single dose of apocynin one hour after glycerol injection. Blood samples were collected for measurement of serum Creatine Kinase (CK), Lactate Dehydrogenase (LDH), Creatinine (Cr) and urea. Reactive Oxygen Species (ROS), 8-Hydroxydeoxy Guanosine (8-OH-dG), Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px), Tumor Necrosis Factor alpha (TNF-α), Myeloperoxidase (MPO) and NADPH oxidase activity were monitored in renal tissue.

Results: Glycerol administration resulted in significant increase of serum CK, LDH, Cr and urea, renal ROS, 8-OH-dG, TNF-α, MPO and NADPH oxidase activity and significantly decreased SOD and GSH-Px activities. Administration of apocynin, both before and after glycerol injection, significantly decreased serum CK, LDH, Cr and urea, renal ROS, 8-OH-dG, TNF-α, MPO and NADPH oxidase activity and significantly increased SOD and GSH-Px activities.

Conclusion: We conclude that administration of apocynin, both before and after glycerol injection, ameliorated renal dysfunction in rhabdomyolysis-induced AKI by inhibiting oxidative stress, inflammatory response.

Key Words: Apocynin – Rhabdomyolysis – Glycerol – Creatine kinase – NADPH oxidase.

Introduction

ACUTE Kidney Injury (AKI) is a common life-threatening disease that places a heavy burden on the health system [1]. It was reported that the mortality rate for this disease is high [2]. Other studies suggest that AKI may be a step in the progression toward chronic kidney disease in human and animals [3,4]. There are many factors that predispose to AKI include hemodynamic instability [5], hypovolemia [6], hypoxia [7], ischemia and reperfusion (I/R) [8] or rhabdomyolysis [9].

Rhabdomyolysis is a syndrome refers to the breakdown of skeletal muscles, which results in the release of potentially toxic compounds, as myoglobin and other intracellular proteins and electrolytes, into the circulation that may affect kidney function [10]. Large numbers of disorders known to cause rhabdomyolysis include intrinsic muscle dysfunctions (including trauma, burns, intrinsic muscle disease, and excessive physical exertion), metabolic disorders, hypoxia, drugs, toxins, infections, temperature extremes and idiopathic disorders [11].

Rhabdomyolysis is often complicated by AKI, electrolyte imbalance, acute cardiomyopathy and disseminated intravascular coagulation [12]. The experimental model for rhabdomyolysis is easily acquired by injecting glycerol intramuscularly into rats [13].

NADPH oxidase (NOX), using NADPH as the source of electrons, catalyzes one electron reduction of molecular oxygen to generate O$_2$ [14], which is a central and initial Reactive Oxygen Species (ROS) molecule and may convert more active and toxic ROS, such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO$^-$), or peroxide nitrite (ONOO$^-$) in the presence of H$^+$, H$_2$O$_2$ and nitric oxide [15]. These O$_2$-derived ROS participating in the inflammatory process [16].

Research has shown that NOX in the kidneys may have a specific pathophysiological role, thus
it is present in different cellular compartments of the kidney [17]. Previous research has shown that the main supplier of ROS in the form of superoxide \( \text{O}_2^- \) in the renal cortex is NOX, whereas in the renal medulla xanthine oxidase also makes similar contribution to \( \text{O}_2^- \) generation along with NOX [18]. Also, NOX has shown to be abundantly present in the macula densa, thick ascending loop of Henle, interstitial cells, blood vessels, glomeruli, and tubules in the kidneys of spontaneously hypertensive rats [17]. Five homologues of the catalytic subunits of NOX have been indentified (NOX 1 - 5) [19].

Apocynin (4-hyroxy-3-methoxy-acetophenone) is an efficient inhibitor of NADPH oxidase [20], and is widely used as antioxidant in research as it a scavenger of \( \text{H}_2\text{O}_2 \) [21]. It was found to inhibit NOX in leukocytes, monocytes and endothelial cells [22]. It was shown that apocynin block NOX4 and NOX5 [23].

Apocynin has been shown to reverse activation of the NADPH oxidase system in rat kidneys induced by hydroxyl-1-proline [17]. In addition, apocynin also exhibits anti-inflammatory effects in previous studies [24]. Some previous studies supported the beneficial role of apocynin against liver and kidney dysfunction [25-28]. It was demonstrated that apocynin can be protective for cisplatin-induced nephrotoxicity in mice [29]. It has protective effects on renal I/R injury [30]. Also, it was reported that apocynin protects the kidney function from toxic effects induced by cyclosporine [31]. However, no study has been conducted to examine the effect of apocynin on rhabomyolysis-induced AKI in albino rats.

So, the aim of the present study was to evaluate the possible protective and therapeutic effect of apocynin on rhabomyolysis-induced AKI in albino rats.

**Material and Methods**

*Animals:*

This study was conducted in accordance with the guidelines for the animal experimental protocols of Tanta Faculty of Medicine from June 2015 to Nov. 2015. It was carried out on forty male albino rats of local strain, 10-12 weeks of age, weighing 200-220g. Rats were housed in clean cages, five rats per each cage, at room with suitable temperature (22±2°C) under controlled 12-12h light dark cycle and had free access to food and water.

*Chemicals:*

Glycerol was purchased from El-Gomhuria Co, Egypt. Apocynin was purchased from Sigma-Aldrich Co.

*Study design:*

Rats were divided into four main groups: Each consisting of 10 animals, the animals were allowed free access to food, but deprived of drinking water for 24h before glycerol injection.

**Group 1 serves as the control group:** The animals were treated with saline intraperitoneal (i.p.), as a vehicle, for 7 days, deprived of water for 24h on the sixth day, then were given saline (10ml/kg intramuscular [i.m.]), half the dose was administered to each hind limb muscle.

**Group 2 is the glycerol group (Gly):** The animals were treated with saline i.p. for 7 days, deprived of water for 24h on the sixth day, then were given a single dose of 50% glycerol 1: 1 (v/v) solution of glycerol and saline (10ml/kg/day i.m.) [32], half the dose was administered to each hind limb muscle.

**Group 3 is apocynin pretreated group (Apo-pretreated):** The animals were treated with apocynin in a dose (20mg/kg i.p.) [30] for 7 days, deprived of water for 24h on the sixth day, then were given glycerol as in Group 2.

**Group 4 is apocynin treated group (Apo-treated):** The animals were treated with saline i.p. for 7 days, deprived of water for 24h on the sixth day, they were given glycerol injection as in Group 2, then given a single dose of apocynin (20mg/kg i.p.) [30] one hour after glycerol injection.

At the end of experiments, at 24h after glycerol injection or saline injection in control, the animals were sacrificed by cervical decapitation. Blood and kidney samples were collected. Blood samples were centrifuged (3000rmp for 10 minutes), and samples were stored at –80°C until assay. For determination of the biochemical parameters in the renal tissues, the isolated kidney was homogenized and was frozen at –80°C for further analysis.

*Estimation of muscle enzymes:*

Serum Creatine Kinase (CK) activity assay kit was purchased from Sigma-Aldrich Co. and was determined according to Apple and Rhodes [33].

*Estimation of tissue damage biomarkers:*

Serum Lactate Dehydrogenase (LDH) activity assay kit was purchased from Sigma-Aldrich Co. and was assessed according to [34].
Estimation of renal function tests:
Serum Creatinine (Cr) and urea were determined colorimetric according to Bauer et al., [35] and kits were purchased from El-Gomhuria Co., Egypt.

Estimation of oxidative stress biomarkers in kidney:
Kidney tissue ROS, 8-hydroxy oxyguanosine (8-OH-dG) were determined by ELISA kits according to Yoshida et al., [36], Glutathione Peroxidase (GSH-Px) were determined colorimetric according the method of Jacobson et al., [37], Superoxide Dismutase (SOD) levels were tested colorimetric according to Kuthan et al., [38]. Kits for oxidative stress biomarkers were purchased from El-Gomhuria Co., Egypt.

Estimation of inflammatory biomarkers in kidney:
Tumor necrosis factor alpha (TNF-α) was determined in the kidney using TNF-α ELISA kit according to Navarro et al., [39], Renal Myeloperoxidase (MPO) was assayed according to the method described by Hillegass et al., [40]. Kits for TNF-α and MPO were purchased from Sigma-Aldrich Co.

Estimation of renal NOX activity:
NOX activity was determined colorimetric in the kidney according to the method described by Vaziri et al., [41]. NOX activity assay kit was purchased from Sigma-Aldrich Co.

Statistical analysis:
The data were shown as the mean ± standard deviation. Data from the study were analyzed using one-way ANOVA. All the analyses were performed using SPSS for windows (Version 16.0).

Results

Effect of apocynin on serum CK:
As shown in Fig. (1A), glycerol injection significantly increased serum CK (1222.50±66.13 U/L) as compared to the control (680.80±33.68 U/L). Both apocynin treatment, for one week prior to glycerol-induced AKI and one hour after glycerol injection, significantly decreased CK as compared to the glycerol group (876.50±80.42 and 870.50±82.14 U/L versus 1222.50±66.13U/L). But the levels of CK, after apocynin treatment, still significantly higher if compared to the control group.

Effect of apocynin on serum LDH:
Compared to control rats, glycerol injection significantly increased serum LDH (224.06±5.88 U/L versus 155.35±7.44U/L). Administration of apocynin, both before and after glycerol injection, significantly decreased LDH as compared to the glycerol group (198.99±5.01 and 200.04±7.36U/L versus 224.06±5.88U/L), however the levels of LDH still significantly higher if compared to the control group Fig. (1B).

Effect of apocynin on kidney function:
Serum urea and creatinine were significantly increased after glycerol injection compared to the control group. While, apocynin treatment, prior to and after glycerol injection, significantly improved the renal functions by decreasing back both creatinine and urea levels as compared to the glycerol group, but their levels still significantly higher if compared to the control rats (Table 1).

Effect of apocynin on renal oxidative stress biomarkers:
These are presented in (Table 2), the levels of ROS, 8-OH-dG significantly increased with glycerol treatment as compared to the normal control rats. While, apocynin treatment, both before and after glycerol injection, showed reduction in ROS, 8-OH-dG levels compared to the glycerol group, but these levels still significantly higher if compared to the control group.

Also, (Table 2) showed that GSH-Px and SOD significantly decreased after glycerol injection as compared to the control group. Apocynin administration, both before and after glycerol injection, significantly increased GSH-Px and SOD levels compared to the glycerol group. It was observed that after apocynin treatment GSH-Px levels still significantly lower than normal control values, but SOD levels return back to the control values.

Effect of apocynin on renal inflammatory biomarkers:
Glycerol injection significantly increased renal TNF-α, MPO levels compared to the control group. The rats treated with apocynin, both prior to and after glycerol injection, showed significant decrease of renal TNF-α, MPO levels compared to the glycerol group, but their levels still significantly higher if compared to the control group Fig. (2A,B).

Effect of apocynin on renal NOX activity:
As shown in Fig. (3), renal NOX activity significantly increased after glycerol treatment (7.1±0.23U/g protein) compared to the control group (1.66±0.46U/g protein). Treatment with apocynin, both before and after glycerol injection, significantly decreased the levels of renal NOX activity as compared to the glycerol group (3.02±0.65 and 3.50±0.65 versus 7.11±0.23U/g protein).
Table (1): Effect of apocynin on serum levels of Cr and urea in rhabdomyolysis-induced AKI in albino rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group</th>
<th>Gly-group</th>
<th>Apo+Gly pretreated group</th>
<th>Apo+Gly treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr (mg/dl)</td>
<td>0.64±0.19</td>
<td>4.10±0.43</td>
<td>2.42±0.32 a b</td>
<td>2.35±0.38 a b</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>17.30±2.75</td>
<td>148.50±5.17 a</td>
<td>97.20±4.89 a b</td>
<td>95.60±5.56 a b</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group</th>
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<th>Apo+Gly treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS (U/mg protein)</td>
<td>50.80±2.25</td>
<td>149.50±3.17 a</td>
<td>91.60±6.48 a b</td>
<td>89.80±5.99 a b</td>
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<tr>
<td>8-OH-dG (ng/mg protein)</td>
<td>2.57±0.35</td>
<td>11.92±0.66 a</td>
<td>8.79±0.47 a b</td>
<td>8.46±0.93 a b</td>
</tr>
<tr>
<td>GSH-Px (mU/mg protein)</td>
<td>98.07±3.49</td>
<td>40.20±7.94 a</td>
<td>78.70±5.77 a b</td>
<td>79.50±7.87 a b</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>0.347±0.007</td>
<td>0.305±0.006 a</td>
<td>0.345±0.006 b</td>
<td>0.343±0.007 b</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.

Fig. (1): Effect of apocynin on serum levels of CK (A), and LDH (B) in rhabdomyolysis-induced AKI in albino rats.

Data are given as mean ± SD. a: p<0.05 vs control group. b: p<0.05 vs Gly-group.

Fig. (2): Effect of apocynin on renal oxidative stress biomarkers in rhabdomyolysis-induced AKI in albino rats.

Data are given as mean ± SD. a: p<0.05 vs control group. b: p<0.05 vs Gly-group.

Fig. (2): Effect of apocynin on renal TNF-α (A), and MPO (B) in rhabdomyolysis-induced AKI in albino rats.

Data are given as mean ± SD. a: p<0.05 vs control group. b: p<0.05 vs Gly-group.
Discussion

This study revealed that glycerol-induced deterioration of renal function in rat kidneys involved oxidative stress and increased inflammatory markers and NOX activity, and for the first time, we investigated the effect of apocynin administration, both before and after glycerol injection, on renal dysfunction caused by intramuscular injection of glycerol, and we found that both pretreatment of animals with apocynin for one week prior to glycerol injection and treatment one hour after glycerol injection significantly reduced renal dysfunction and improved the alterations observed with glycerol injection.

Intramuscular injection of glycerol in rats results in rhabdomyolysis-induced AKI [42]. CK is the most sensitive damage index for muscle cells [43]. In this study, serum levels of both CK and LDH significantly increased after i.m. injection of glycerol which confirmed muscle damage. Also, the results of the present study showed renal dysfunction in the form of significant increase of urea and creatinine levels.

The mechanism of rhabdomyolysis-induced AKI results from the lysis of myocytes and release of large amount of myoglobin into the circulation, then the released myoglobin is deposited in the kidney, causing renal tubular obstruction and necrosis accompanied by intense renal vasoconstriction with subsequent renal damage [44].

In the present study, treatment of rats with apocynin, both 7 days prior to glycerol injection and one hour after glycerol injection, significantly decreased serum CK and LDH and renders rats less susceptible to kidney damage induced by glycerol injection. This protection was evidenced in the serum as the elevated levels of both urea and creatinine were markedly lowered below those elicited in the glycerol group.

Glycerol-induced renal damage was accompanied by oxidative stress [45]. The present study showed that the levels of ROS, 8-OH-dG in renal tissue significantly increased, whereas the activity of GSH-Px and SOD significantly decreased. In accordance to those results, previous reports that have shown that exercise for long distance running may cause lipid peroxidation damage in the skeletal muscle and kidney [46]. The mechanism by which glycerol can cause renal oxidative stress can be explained by myoglobin deposition in the kidney is associated with the production of ROS and free radicals in the mitochondria, which initiate lipid peroxidation reactions and depletion of antioxidant enzymes [47].

Another mechanism by which glycerol can cause oxidative stress is that glycerol, acting as a scavenger molecule, produces secondary scavenger-derived free radicals capable of damaging DNA [48]. In addition, considering that the kidney is responsible for the metabolism of 20% of all glycerol, the glyceraldehydes produced may autoxidized in the presence of oxygen, yielding superoxide radical with accumulation of hydrogen peroxide [48]. Also, a major source of ROS is NOX activation [49] as proved in the results of this work.

In the present study, apocynin, in both Groups 3 and 4, significantly decreased the levels of renal ROS, 8-OH-dG with concomitant significant increase of GSH-Px and SOD in renal tissue. These results are in line with previous results suggested that apocynin inhibited free radical generation and increasing antioxidant defense in testicular tissues against I/R [50]. The mechanism by which apocynin could decrease the tissue oxidative damage in this model of AKI, may be due to its direct antioxidant effect [21]. But, also, it could be due to inhibition of NOX activity [22], and lowering the production of inflammatory mediators such as TNF-α [51] as proved in the results in this work.

The results of the current study demonstrated significant increase of the levels of renal TNF-α and MPO after glycerol injection, which may implicate increased inflammatory process. The mechanism by which glycerol triggers release of renal TNF-α have yet to be determined, but it may be explained by oxidative injury of the renal tissues that promotes release of TNF-α [52]. The results of the present study demonstrated that
apocynin significantly reduced the renal TNF- \( \times \) level. This in accordance with previous results found that apocynin prevented the increase of hepatic TNF- \( \times \) in fructose diet-fed rats [25]. Also, Meng et al., proved that apocynin administration reduced the circulating level of TNF- \( \times \) in high fat diet-fed rats [81].

Also, in the present study, the elevated MPO activity in renal tissue indicates the contribution of neutrophil infiltration in glycerol-induced renal injury. This may be due to oxidative stress which accompanied by neutrophil infiltration [53]. Administration of apocynin, both before and after glycerol injection, significantly reduces the MPO activity. These results suggest that the mechanism of the protective and therapeutic effect of apocynin involves the inhibition of inflammatory cell infiltration [54].

Finally, the present study proved that the NOX activity in kidney was significantly increased in glycerol injected rats. This was in accordance with Newaz et al., who demonstrated that increased free radical generation in model of glycerol-induced ARF was associated with an increased NOX activity [58]. While in the present study, apocynin injection, both before and after glycerol injection, significantly decreased the activity of NOX in renal tissue. The mechanism by which apocynin affects NOX activity is not totally known, but the effect of apocynin could be ascribed, at least partially, to the inhibition of different NOX subunits at the membrane level [22]. Also, it was suggested that apocynin prevented the enhancement of NOX gene and protein expression [25].

**Conclusion:**

We suggested that apocynin ameliorated renal dysfunction in rhabdomyolysis-induced AKI by inhibiting oxidative stress, inflammatory response and NOX activity. So, apocynin can be selected as a potential therapeutic agent of clinical acute renal injury by rhabdomyolysis. Although it is difficult to administer apocynin before rhabdomyolysis is clinically diagnosed, it may be beneficial if it is administered at an early phase of rhabdomyolysis. Also, it can be beneficial pretreatment in sport medicine and military medicine with expected exertional rhabdomyolysis.

**References**


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الملخص العربي

الهدف: تهدف هذه الدراسة إلى التحقيق في التأثير الوظيفي والعلاجي المحتمل لأبيسينين على إصابة الكلى الحاد الناجم عن انحلال الريبيدات في الجدراان البيضاء.

طرق البحث: تم تقسيم 40 ذكر الجدراان البيضاء بشكل عشوائي إلى 4 مجموعات: مجموعة 1 تمثل المجموعة الضابطة. وأعطيت المجموعة 2 50% جلسرين (10مل/كجم، عن طريق الحقن العضلي). وأعطيت مجموعة 3 10 جلسرين بعد 7 أيام من المعالجة بالبيسينين (0.1جم/كجم/يوم عن طريق الحقن داخل الصلاب). وأعطيت مجموعة 4 جرعة واحدة من أبيسينين (0.2جم/كجم) المكمل من حقن داخل الصلاب وذلك بعد سبعة واحدة من حقن الجلسرين. وفي نهاية التجربة، تم ذبح الفئران وجمع عينات الدم لقياس الكرياتين في الدم، السيراميد، الكرياتين في الدم، الأوكسيجين، الفيتيلاك، والأشعة. كما تم قياس مستوى الأكسدة في أنبسة الكلى وكذلك تقياس كل من ROS, آفيرغينوكس (8-OH-dG). ونظام الأكسدة الكلي. كما تم قياس نشاط NADPH أوركسيد في أنبسة الكلى.

النتائج: أظهر النتائج أن الحقن بالجلسرين أدى إلى زيادة ذات دلالة إحصائية في كل من الكرياتين في الدم، السيراميد، أوركسيد NADPH، آفيرغينوكس (8-OH-dG)، مستويات كل من ROS، ونظام أكسدة الكلي. كما تم قياس التركيب النشاطي بشكل ملحوظ للذات كل من الديميسنت الأكسدة والتوليميكتون بيركسيد في أنبسة الكلى.

وقد وجد أن التأثيرات الباراتينين قبل وبعد حقن الجلسرين على حد سواء أدى إلى انخفاض في كل من الكرياتين في الدم، السيراميد، أوركسيد NADPH، مستويات كل من ROS، ونظام أكسدة الكلي. كما تم قياس النشاط بشكل ملحوظ للذات كل من الديميسنت الأكسدة والتوليميكتون بيركسيد في أنبسة الكلى.

الخلاصة: الجلديين باراتينين قبل وبعد حقن الجلسرين، أدى إلى تحفيز إصابة الكلى الحاد الناجم عن انحلال الريبيدات وذلك عن طريق NADPH أوركسيد في أنبسة الكلى، تثبيت الأكسدة، الاستجابة التهابية والنشاط في أنبسة الكلى.