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Effect of phosphodiesterase-5 inhibition on joint and muscle damage in rats with adjuvant arthritis

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Background/aim: This study was designed to examine the effect of tadalafil, a phosphodiesterase (PDE)5 inhibitor, on the severity of joint and muscle damage in rats with adjuvant-induced arthritis (AA).

Materials and methods: AA was induced by intradermal inoculation into right hind paw of male Sprague Dawley rats (300–450 g) with complete Freund’s adjuvant (CFA; 0.1 mL). AA rats were treated with either tadalafil (10 mg/kg; per oral) alone or along with the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 mg/kg; intraperitoneally). After decapitation on day 16, trunk blood was collected for total oxidant status (TOS) and total antioxidant capacity (TAC) assays. The left metatarsophalangeal joint and gastrocnemius muscle were excised for microscopic examination. Muscle samples were also evaluated in terms of malondialdehyde (MDA), glutathione, and chemiluminescence (CL) levels.

Results: In tadalafil-treated AA rats, metatarsophalangeal joints revealed regular morphology of the cartilage with slight destruction and less inflammatory cell infiltration and vascularization in comparison to the controls (microscopic score: 1.17 ± 0.31 vs. 4.17 ± 0.79; P < 0.01). AA rats presented increased gastrocnemius muscle MDA, glutathione, and CL levels compared to the controls (P < 0.01, for MDA; P < 0.05, for glutathione; P < 0.05 for CL). Tadalafil attenuated the increase in CL levels (P < 0.01, for luminol and P < 0.001, for lucigenin). Serum TOS showed significant reductions by tadalafil.

Conclusion: The long-acting PDE5 inhibitor tadalafil provides partial protection in a rat model of CFA-induced arthritis possibly via suppression of oxidant generation.

Key words: Arthritis, phosphodiesterase-5, rat, tadalafil

1. Introduction
Rheumatoid arthritis is a chronic, inflammatory, and systemic autoimmune disorder characterized by synovial inflammation and destruction of cartilage and bone. The etiopathogenesis of the disease includes the inflammatory cytokines (e.g., tumor necrosis factor-α, interleukin-1β, and interleukin-6) and inflammatory mediators (e.g., cyclooxygenases and lipoxygenases) (1). Studies have shown the presence of neutrophils in the synovial fluid and on the pannus-cartilage interface in arthritis (2,3). Isolated neutrophils from arthritic patients showed priming that may be related to the presence of pro-inflammatory cytokines in the synovial fluid (4). Thus, the activated neutrophils may contribute to joint destruction through the release of reactive oxygen species (ROS).

Cachexia has also been reported in rheumatoid arthritis patients and seems to be an important contributor in increasing morbidity and mortality (5). Although experimental chronic arthritis induces anorexia, the weight loss and muscle wasting observed in animals with arthritis were not found to be associated with decrease in food intake. Instead, the muscle wasting was mostly due to systemic inflammation (6).

Phosphodiesterases (PDEs) are metallohydrolases that catalyze the breakdown of cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) into the inactive 5’-AMP or GMP, thereby regulating the duration and amplitude of cyclic nucleotide signaling. PDEs comprise a 21-gene superfamily categorized into 11 families (PDE1–PDE11) (7). PDE5 is widely expressed in the corpora cavernosa of the penis, systemic arteries and veins, the pulmonary arteries, the myocardium, the skeletal muscles, and the platelets (8,9). Potent and selective PDE5 inhibitors have
been approved for therapeutic use for erectile dysfunction (10), pulmonary hypertension (11,12) and Raynaud’s phenomenon (13). Previous studies reported that a long-acting PDE5 inhibitor, tadalafil, is beneficial in ischemic injury of neurons, ovary, myocardium, kidney, and liver via preventing ROS damage, lipid peroxidation, and apoptosis and restoring antioxidant levels (14–18). Chronic treatment of diabetic rats with tadalafil improved redox signaling by enhancing the antioxidant enzyme glutathione S-transferase kappa-1 and downregulated redox regulatory chaperones, heat shock protein 8, and 75 kDa glucose regulatory protein (19). Koka et al. (16) demonstrated that chronic treatment with tadalafil attenuated oxidative stress and improved mitochondrial integrity in mice with type 2 diabetes. In another study, tadalafil at a dose of 10 mg/kg ameliorated circulating inflammatory cytokines, reversed oxidant/antioxidant dysfunction, and thus protected renal tissue from Escherichia coli-induced acute pyelonephritis in rats (20). In 36 patients (age range: 37–59 years) with diabetes. In another study, tadalafil at a dose of 10 mg/kg ameliorated circulating inflammatory cytokines, reversed oxidant/antioxidant dysfunction, and thus protected renal tissue from Escherichia coli-induced acute pyelonephritis in rats (20). In 36 patients (age range: 37–59 years) with clinically documented mild to severe erectile dysfunction, tadalafil citrate protected the cardiovascular system by reducing serum levels of oxidative stress (21).

The present study was designed aiming to examine the effect of tadalafil on the severity of joint and muscle damage in rats subjected to adjuvant-induced arthritis (AA).

2. Materials and methods

2.1. Animals and chemicals

Male Sprague Dawley rats (300–450 g) were allowed to acclimatize for a week before the experiments were started. They were housed under standard laboratory conditions (room temperature: 20–26 °C; relative humidity: 40%–60%; free access to water and food) and maintained on a 12-h light–12-h dark cycle. The study protocol was approved by Marmara University School of Medicine, Animal Care and Use Committee. The experimental procedures were conducted in accordance with the Guide to the Care and Use of Laboratory Animals.

2.2. Experimental protocol

The rats were randomly allocated into four groups (n = 8 per group). To induce AA, the rats were inoculated intradermally into the plantar surface of the right hind paw with 0.1 mL of complete Freund’s adjuvant (CFA) (Sigma, St. Louis, MO, USA) containing 10 mg/mL of heat-killed Mycobacterium tuberculosis suspended in paraffin oil (AA group) (22). The control rats were injected with paraffin oil (0.1 mL) (control group). In the treatment groups, the rats with AA were treated with tadalafil (Tork, 20 mg per tablet, Bilim İlaç, Turkey) (10 mg/kg; per oral) once daily between day 5 and day 15 after immunization (AA + tadalafil group). In another group, the rats with AA received the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 mg/kg; intraperitoneally) (Sigma) 30 min before tadalafil injection on day 15 (AA + ODQ + tadalafil group). The doses of tadalafil and ODQ used in this study were demonstrated to be effective in previous studies (23,24). Tadalafil tablets were crushed and suspended in water. ODQ stock solution was prepared in dimethyl sulfoxide 30% v/v in saline.

All rats were decapitated on day 16. Blood samples were collected and centrifuged at 3000 rpm for 15 min. Serums were stored at –80 °C for the assessment of total oxidant status and antioxidant capacity. The left hind paw was amputated at the ankle. The left metatarsophalangeal joint and gastrocnemius muscle were dissected. The gastrocnemius muscle was weighed. Both joint and muscles were fixed in 10% buffered formaldehyde for histopathological evaluation. Gastrocnemius muscle samples were stored at –80 °C for subsequent measurement of malondialdehyde (MDA) level and glutathione content. Formation of ROS in muscle samples was monitored using the Chemiluminescence (CL) method.

2.3. Evaluation of arthritis

Arthritis development was evaluated daily, as described previously (26). Briefly, the hind ankle circumference was determined by measuring the laterolateral diameter (a) and the anteroposterior diameter (b) with digital calipers and was calculated using the following formula (25):

\[
\text{Circumference (mm)} = 2\pi \left(\sqrt{a^2 + b^2}\right)/2
\]

2.4. Histological examination of the metatarsophalangeal joint and gastrocnemius muscle

Metatarsophalangeal joint and gastrocnemius muscle samples were placed in 10% buffered formaldehyde. Joint samples were kept in a decalcifier solution (Osteomoll, Merck KGaA, Darmstadt, Germany) for 5 days. Both joint and muscle samples were dehydrated in ascending alcohol series (70%, 90%, 96%, and 100%) and embedded in paraffin. For each animal, four randomly taken tissue sections (5 µm) were stained with hematoxylin and eosin and Masson’s trichrome. From each section, a minimum of three areas were randomly selected for histopathological examination and visualized with a microscope (Olympus BX51, Tokyo, Japan).

The metatarsophalangeal joints were histologically scored for inflammation, cartilage damage, pannus formation, and bone resorption using the criteria described in Table 1 (26).

Gastrocnemius muscle samples were examined considering disorganization and degeneration of muscle fibers, and inflammatory cell infiltration.

Histopathological examinations were performed by experienced histologists who were unaware of the treatment groups.
2.5. Measurement of malondialdehyde (MDA) and glutathione levels
Gastrocnemius muscle MDA and glutathione levels were measured in samples homogenized in 10 volumes of ice-cold 10% trichloroacetic acid by spectrophotometric methods, as described previously (27,28).

2.6. CL assay
Luminescence of the gastrocnemius muscle homogenates was recorded using a Mini Lumat LB 9506 luminometer (EG&G Berthold, Germany) in the presence of luminol or lucigenin 0.2 mM each. The results were expressed as area under the curve (AUC) of relative light unit (rlu) for 5 min per mg tissue (29).

2.7. Serum total oxidant status (TOS) and total antioxidant capacity (TAC) assays
Measurement of TOS level is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidative species in acidic medium and the measurement of the ferric ion by xylenol orange (30). As described by Erel (31), TAC assay is based on the bleaching of the characteristic color of the more stable 2,2’-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) radical cation by antioxidants. Measurements were performed using commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey) via an autoanalyzer (PG Instruments Ltd, Leicestershire, UK). TOS and TAC results are expressed as mmol H₂O₂ equivalent per liter of serum and mmol Trolox equivalent per liter of serum, respectively.

2.8. Statistical analysis
Data are expressed as mean ± SEM. The histological data were compared by Mann–Whitney U nonparametric test. Other parameters were compared using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison tests. Values of P < 0.05 were regarded as significant. Calculations were done using the statistical analysis package Instat (GraphPad Software, San Diego, CA, USA).

3. Results
3.1. Evaluation of arthritis
Evaluation of the arthritis severity on day 16 revealed significantly increased ankle circumference in AA rats compared to the controls (34.76 ± 0.93 mm vs. 31.28 ± 0.14 mm; P < 0.01). Treatment of AA rats with tadalafil failed to protect the tissue from edema formation as the ankle circumference was even higher (37.79 ± 0.60 mm; P < 0.05) compared to the untreated AA group.

No significant difference was observed between the experimental groups in terms of gastrocnemius muscle weight (data not shown).

3.2. Microscopic evaluation of the metatarsophalangeal joint and gastrocnemius muscle
Histopathological examination of the metatarsophalangeal joint sections showed thinning of articular cartilage, disorganization of cartilage surface, increased inflammatory cell infiltration, desquamation of synovial epithelium, and increased vascularization in the AA group. In the tadalafil-treated AA group, the joint samples revealed regular morphology of the cartilage with slight destruction, with less inflammatory cell infiltration and vascularization in comparison to the untreated group. ODQ given prior to tadalafil did not change the extent of joint injury in comparison to the tadalafil-treated AA group (Figure 1).

Table 1. Histopathology scoring criteria used for the evaluation of metatarsophalangeal joints.

<table>
<thead>
<tr>
<th>Score</th>
<th>Inflammation</th>
<th>Pannus formation</th>
<th>Cartilage damage</th>
<th>Bone resorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None present</td>
<td>None present</td>
<td>None present</td>
<td>None present</td>
</tr>
<tr>
<td>1</td>
<td>Minimal infiltration in periarticular tissue</td>
<td>Minimal infiltration of pannus in cartilage and subchondrial bone</td>
<td>Minimal damage</td>
<td>Small areas of resorption</td>
</tr>
<tr>
<td>2</td>
<td>Mild infiltration (&lt;1/4 of tibia at edges)</td>
<td>Focal chondrocyte loss</td>
<td>More numerous areas of resorption</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Moderate inflammation with moderate edema (1/4 to 1/3 of tibia affected)</td>
<td>Mid zone chondrocyte loss</td>
<td>Resorption of medullary trabecular and cortical bone</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Marked infiltration with marked edema (1/2 to 3/4 of tibia affected)</td>
<td>Deep zone chondrocyte loss</td>
<td>Full thickness defects in cortical bone, marked loss of medullary bone, 1/2 to 3/4 of tibia affected</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Severe infiltration with severe edema (&gt;3/4 of tibia affected)</td>
<td>Severe chondrocyte loss</td>
<td>Full thickness defects in cortical bone, marked loss of medullary bone, &gt;3/4 of tibia affected</td>
<td></td>
</tr>
</tbody>
</table>
In agreement with these data, in the tadalafil-treated AA group, the microscopic score was lower compared to that of the untreated AA group (1.17 ± 0.31 vs. 4.17 ± 0.79; *P* < 0.01). ODQ administration did not change tadalafil’s effect on this parameter (1.15 ± 0.34) (Table 2).

Gastrocnemius muscle sections demonstrated regular morphology in all experimental groups (Figure 2).

### 3.3. Gastrocnemius muscle MDA, glutathione, and CL levels

The AA group presented increased gastrocnemius muscle MDA (*P* < 0.01) and glutathione (*P* < 0.05) levels compared to the control group. In the tadalafil-treated AA group, these parameters did not seem to change significantly compared to the untreated AA group. Muscle MDA level showed further elevations when ODQ was administered prior to tadalafil (*P* < 0.01) (Table 3).

As demonstrated in Figures 3 and 4, gastrocnemius muscle luminol- and lucigenin-enhanced CL levels showed significant elevations in the AA group (13.17 ± 2.44 rlu/mg and 10.69 ± 1.43 rlu/mg, respectively) compared to the control group (5.30 ± 0.62 rlu/mg and 6.00 ± 0.95 rlu/mg, respectively) (*P* < 0.01, for luminol; *P* < 0.05, for lucigenin). Tadalafil treatment was effective to reduce these values back to control levels (4.48 ± 1.12 rlu/mg and 3.04 ± 0.33 rlu/mg) (*P* < 0.01, for luminol; *P* < 0.001, for lucigenin). ODQ given prior to tadalafil did not change the effect of tadalafil on these parameters.

### 3.4. Serum TOS and TAC data

Although the increase in serum TOS level in the AA group (37.85 ± 5.63 mmol H₂O₂ Equiv/L) did not reach a statistically significant level compared to the control group, tadalafil administration to AA rats decreased serum TOS levels (21.16 ± 2.43 mmol H₂O₂ Equiv/L). Serum TOS levels were comparable among tadalafil- or ODQ + tadalafil-treated AA rats (Table 4).

There were no significant differences between the groups in terms of serum TAC levels (Table 4).

### 4. Discussion

CFA-induced rat arthritis shows clinical and pathological similarity to human rheumatoid arthritis and is the
most widely used animal model (32). Our study results demonstrated damage to the metatarsophalangeal joint and increased ROS production in the gastrocnemius muscle following intradermal inoculation of CFA into the right hind paw of rats and partial protection by tadalafil, a long-acting PDE5 inhibitor, given at a dose of 10 mg/kg for 10 days. Evaluation of the left ankle at microscopic and microscopic levels following CFA inoculation revealed

**Figure 2.** Micrographs illustrating the histological appearances of gastrocnemius muscle samples from the experimental groups. The control group (A), AA group (B), AA + tadalafil group (C), and AA + ODQ + tadalafil group (D) reveal regular striated muscle morphology in terms of muscle organization and fibers. H&E staining. Original magnification 100×; insets: 400×. AA, adjuvant arthritis; ODQ, 1H-1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.

**Table 3.** Gastrocnemius muscle malondialdehyde (MDA) and glutathione levels of the experimental groups. Data are expressed as mean ± SEM.

<table>
<thead>
<tr>
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<th>MDA (nmol/g)</th>
<th>Glutathione (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (n = 8)</td>
<td>1.52 ± 0.27</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>AA group (n = 8)</td>
<td>6.60 ± 1.26**</td>
<td>0.75 ± 0.08*</td>
</tr>
<tr>
<td>AA + treatment groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tadalafil (n = 8)</td>
<td>6.86 ± 1.13</td>
<td>0.65 ± 0.05</td>
</tr>
<tr>
<td>ODQ + tadalafil (n = 8)</td>
<td>25.81 ± 5.98 +++</td>
<td>0.65 ± 0.14</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 vs. control group, ***P < 0.01 vs. AA group, ****P < 0.01 vs. AA + tadalafil group. AA, adjuvant arthritis; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.
increased ankle circumference (indicating edema), thinning of articular cartilage, disorganization of cartilage surface, increased inflammatory cell infiltration, desquamation of synovial epithelium, and increased vascularization. Recent research on CFA-induced arthritis suggests a role of oxidative stress in the pathophysiology (33). The role of ROS—derived from macrophages, lymphocytes, neutrophils, and endothelial cells at the site—has been studied in the pathophysiology of inflammatory synovitis (34). ROS comprise the cell membrane or organelles via oxidation of polyunsaturated fatty acids. Lipid peroxidation causes loss of membrane fluidity and impairs ion transport and membrane integrity, leading to loss of cellular functions (35). Prior studies reported increased lipid peroxidation in patients with rheumatoid arthritis (36) and in plasma of rats with CFA-induced arthritis (37). MDA is a major oxidative degradation product of membrane unsaturated fatty acid and is used as an indicator of lipid peroxidation. In our study, we observed increased MDA levels in the gastrocnemius muscle samples from rats with AA.

The body possesses antioxidant defenses, repair mechanisms, physical defenses, and preventive mechanisms against ROS-induced oxidative stress. Endogenous glutathione acts as a free radical scavenger participating in the metabolism and detoxification of electrophilic drugs, antioxidant defense, and maintenance of thiol redox status (38). In our study, rats with AA had higher gastrocnemius muscle glutathione levels in comparison to the controls. Increased levels of GSH in AA might be a defensive response to excessive formation of ROS and cellular lysis associated with arthritis progression (39).

CL is a simple and a reproducible technique for demonstrating the generation of oxidants in tissues. Among the two CL probes, luminol detects H$_2$O$_2$, hydroxyl radical, hypochlorite, peroxy nitrite, and lipid peroxyl radicals and lucigenin is particularly sensitive to superoxide radical (40). It is well known that superoxide radicals play a role in the degradation of collagen, resulting in the acceleration of inflammatory reactions and damage to joints through the activation of cells such as neutrophils (41). During the course of inflammation, an increase in ROS and superoxide radicals contributing to lipid peroxidation was demonstrated in synovial joints (42). In the present study, we observed increased luminol- and lucigenin-enhanced CL levels in the gastrocnemius muscle of AA rats. Thus, increased levels of MDA and glutathione correlate with increased ROS generation in the gastrocnemius muscle of AA rats. This demonstrates the presence of oxidant stress in the muscle tissue in spite of regular morphology in AA rats.

Although serum concentrations of different oxidant species and antioxidant molecules could be measured by direct or indirect methods separately, TOS and TAC assays are generally preferred as they provide an overall measurement of cumulative oxidative and antioxidant status. In the present study, we used a colorimetric and automated method for the measurement of TOS and TAC, as described by Erel (30,31). In our study, the AA group presented significantly higher serum TOS values in comparison to the control group. This finding is in agreement with the gastrocnemius muscle MDA and CL data.
There is limited information about the effect of PDE5 inhibitors on oxidative mechanisms. Sildenafil has been shown to inhibit ROS formation and protect against oxidative stress. In the study by Perk et al. (43), a single 100 mg/kg dose of sildenafil resulted in a significant increase in erythrocyte superoxide dismutase and catalase activities in healthy men. In a rat model of bleomycin-induced lung fibrosis, sildenafil reversed tissue MDA levels, myeloperoxidase activity, and preserved glutathione (44). Similarly, sildenafil citrate (5 mg/kg per day) for 3 days showed significant protection in a rat acetic acid-induced colitis model via its actions on oxidant/antioxidant status (45). Tadalafil has a longer half-life, and greater selectivity for PDE5 than sildenafil (18). In a recent study by Bektas et al. (18), tadalafil (10 mg/kg) prevented ROS damage, lipid peroxidation, hepatocyte necrosis, and apoptosis in rat liver ischemia/reperfusion injury and minimized liver damage. In a zymosan-induced rat model of arthritis, tadalafil (0.02–0.5 mg/kg, per oral) dose-dependently decreased neutrophil influx and tumor necrosis-alpha release into the synovial cavity (46). In our study, evaluation of the inflamed joint at microscopic level showed protection by tadalafil (10 mg/kg; per oral) with no significant recovery in joint edema. Additionally, tadalafil administration to AA rats did not cause a significant change in muscle MDA and glutathione but reduced CL and plasma TOS.

A clinical study on patients with erectile dysfunction demonstrated the beneficial action of tadalafil on the cardiovascular system via restoring serum TOS and TAC levels. As suggested by the authors, this effect might be due to prevention of the activity and expression of nicotinamide adenine dinucleotide phosphate oxidase by enhancing cyclic GMP levels, which would reduce the formation of ROS while increasing antioxidant enzymes (43).

In our study, we also examined whether the effects of tadalafil would be modified by cGMP blockade. However, the guanylate cyclase inhibitor ODQ did not change tadalafil's actions on AA-induced joint and muscle damage. Thus, the effects of tadalafil in this model of arthritis do not seem to be mediated by cGMP.

An in vitro study in our laboratory examined the superoxide radical scavenging ability of tadalafil in a xanthine/xanthine oxidase assay. In this study, tadalafil reduced lucigenin-enhanced luminescence at a dose 0.25 mg/mL (601.3 ± 47.9 rlu vs. 1029.0 ± 124.0 rlu; P < 0.05) and ODQ did not change this effect (unpublished results).

In conclusion, the results of our study demonstrate damage to the metatarsophalangeal joint and increased ROS production in the gastrocnemius muscle in a rat model of CFA-induced arthritis and partial protection by the long-acting PDE5 inhibitor tadalafil possibly via suppression of ROS generation.

Acknowledgments
The authors thank Marmara University Scientific Research Project Commission for the financial support of this study (SAG-C-DRP-121214-0380).

References

### Table 4. Serum total oxidative status (TOS) and total antioxidant capacity (TAC) levels of the experimental groups. Data are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>TOS (mmol H₂O₂ Equiv/L)</th>
<th>TAC (mmol Trolox Equiv/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (n = 8)</td>
<td>33.97 ± 3.87</td>
<td>3.72 ± 0.20</td>
</tr>
<tr>
<td>AA group (n = 8)</td>
<td>37.85 ± 5.63</td>
<td>3.22 ± 0.17</td>
</tr>
<tr>
<td>AA + treatment groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tadalafil (n = 8)</td>
<td>21.16 ± 2.43*</td>
<td>3.67 ± 0.16</td>
</tr>
<tr>
<td>ODQ + tadalafil (n = 8)</td>
<td>12.54 ± 3.66**</td>
<td>3.27 ± 0.30</td>
</tr>
</tbody>
</table>

AA, adjuvant arthritis; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one.
**P < 0.01 vs. control group. *P < 0.05, **P < 0.01 vs. AA group.


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