Effect of monensin, a Na\textsuperscript{+}-specific carboxylic ionophore on the oxidative defense system in rat testis

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Abstract:
The effect of monensin, a Na\textsuperscript{+}-specific ionophore on the oxidative defense system in rat testis was studied. Monensin mixed in the animal diet was administered at the dose levels of 2.5, 5 and 10 mg/kg b.w. to Wistar rats for a period of 67 days. A marked inhibition in the activities of different oxidative defense enzymes such as catalase, glutathione peroxidase, glutathione-S-transferase, superoxide dismutase and glutathione reductase was noticed, which indicates the possible involvement of free radicals in the antispermatogenic effects of monensin in rat testis. This was further substantiated by a significant increase in the generation of lipid peroxides along with the depletion of reduced glutathione. The drug treatment resulted in a significant change in apoptotic cell death as seen by an elevated fragmentation in the testicular genomic DNA. Monensin treatment also resulted in marked degenerative changes in the histoarchitecture of testis, such as depletion of different germ cell populations, vacuole formation and disorganization of seminiferous tubules. The results are indicative of the potential antispermatogenic effects of monensin in the rat.

Key words:
monensin, oxidative damage, DNA fragmentation, rat testis, antispermatogenic, histoarchitecture

Introduction

Monensin, a Na\textsuperscript{+} ionophore, has been widely used as a biochemical tool to study the function of Golgi apparatus [21]. The ionophore is derived from Streptomyces cinnamonensis and used as a coccidiostatic in avian species and as a growth promoter in cattle. It easily forms lipophilic complexes with other ions (monovalent cations) and results in an influx of Na\textsuperscript{+} with corresponding efflux of H\textsuperscript{+} and K\textsuperscript{+} leading to an increase in intracellular Ca\textsuperscript{2+} [8]. These cation imbalances are supposed to cause a number of major biochemical and histological changes including swelling of mitochondria, decrease in ATP production, lipid peroxidation and loss of cell membrane integrity [21]. Several cases of monensin toxicosis have been reported in different animal species [9, 31]. Metabolic studies with \textsuperscript{14}C monensin in several food producing species and in the rat, indicated that the ionophore is biotransformed extensively to a large number of metabolites (> 50) that are qualitatively but not quantitatively similar among different species [8]. Monensin also appears to be a substrate of P-450-dependent monoxygenases in rat liver microsomes [5]. One of the first subcellular effects observed after the topical application of monensin was vacuolization of Golgi body cisterns where, cells usually show deviation in Golgi structure and function after their exposure to monensin [21]. Monensin has been known to alter the
ion gradients and intracellular pH, which regulate many cellular functions, like cell differentiation, proliferation and apoptosis [34]. It has also been reported that this ionophore induces apoptosis in different cancer cell lines [24–27].

Monensin has been shown to inhibit spermatogenesis in rats [3, 20]. Although it seems that disturbances of Golgi structure and function plays an important role in the antifertility effects of monensin, but investigations of other phenomena, like oxidative damage and apoptosis have not been studied. As the interaction of the reactive oxygen species (ROS) with the ion transport mechanism plays an important part in the regulation of different cellular functions [15], the present study was planned to evaluate the oxidative damage produced by monensin in rat testis.

**Materials and Methods**

**Chemicals**

All the chemicals used in different procedures including enzymatic analysis, histology and DNA fragmentation test were of analytical grade, purchased locally from reputed firms. Monensin was purchased from Sigma Chemical Co, St. Louis, Mo, USA.

**Animals and treatment**

Male Wistar rats in the weight range of 150–200 g were procured from the Central Animal House of Panjab University, Chandigarh. They were acclimatized in the departmental animal house for one week before the drug treatment, and were maintained in a well-ventilated room and given pellet feed and water ad libitum. All procedures were done in accordance with the ethical guidelines for care and use of laboratory animals and were approved by the local ethical committee for experimental animal care. The animals were randomly assigned into four groups, monensin was mixed in the animal diet at different concentrations. Group I consists of control animals, while group II, III, IV animals were administered with 2.5, 5 and 10 mg monensin/kg, respectively. The animals were sacrificed after 67 days of treatment and the testes were processed for biochemical, histological and DNA fragmentation analysis.

**Biochemical analysis**

A 10% homogenate of testis was made in 0.1 M Tris buffer (pH 7.4) with a motor-driven homogenizer. The homogenate was centrifuged at 10,000 × g and the post mitochondrial supernatant was used in all the biochemical analyses.

Catalase activity was determined by the method of Luck [18]. Glutathione peroxidase was estimated by the method of Paglia and Valentine [23], while glutathione-S-transferase was evaluated according to the method of Habig et al. [10]. Method of Kono [14] was applied to calculate the activity of superoxide dismutase, while glutathione reductase was estimated by the method of Massey and Williams [19]. Reduced glutathione was quantified by the method of Moren et al. [22]. Lipid peroxidation levels were measured with the method of Beuge and Aust [4]. Proteins were calculated according to the method of Lowry et al. [17].

**DNA fragmentation analysis**

DNA was isolated from the 10% testis homogenate by the standard procedure. Briefly, the homogenate was treated with 10% SDS and proteinase K followed by CTAB/NaCl treatment. DNA was separated on a 2% agarose gel (50 V) and stained with ethidium bromide. The gel was viewed under ultraviolet light to observe fragmentation of DNA and then photograph was taken.

**Histology**

For histological studies, the tissue was fixed in Bouin’s fixative. The paraffin sections were cut 5 µm and stained with hematoxylin and eosin.

**Statistical analysis**

Data were analyzed using Student’s t-test [11] and are expressed as the mean ± SD.

**Results**

An overall inhibition in the activity of catalase was observed in all the treated groups in comparison to the control (Tab. 1). The lowest dose (2.5 mg/kg, group
II) caused a reduction by 35.3% (p < 0.01), whereas the activity was inhibited by up to 67% (p < 0.001) and 72.4% (p < 0.001), respectively, in groups III and IV (5 and 10 mg/kg). There was a marked decrease in the activity of glutathione peroxidase (p < 0.001) in groups III and IV. Similarly, a significant decline (p < 0.01) in the activity of glutathione-S-transferase was observed in groups III and IV animals. On the other hand, significant inhibition (p < 0.001) in SOD activity was observed in groups II, III and IV. Glutathione reductase was found to be significantly decreased (p < 0.05) in group II animals and similarly groups III and IV were registered with a decrease in enzyme activity (p < 0.001). The content of reduced glutathione was depleted significantly (p < 0.05) in group II, while groups III and IV have shown a marked depletion in GSH content (p < 0.001). The level of lipid peroxidation was significantly elevated in all the treated groups, an increase by 31% (p < 0.01) was noticed in group II animals. However, the elevation in groups III and IV was by up to 57.4% (p < 0.001) and 72.4% (p < 0.001), respectively (Tab. 2).

From the DNA studies of testis on agarose gel electrophoresis, it was observed that monensin has caused

### Tab. 1. The effect of various doses of monensin on the activities of oxidative stress-related enzymes in the testis of Wistar rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Catalase (µmoles of H₂O₂ decomposed/min/mg protein)</th>
<th>Glutathione peroxidase (µmoles of NADPH oxidized/min/mg protein)</th>
<th>Glutathione-S-transferase (µmoles of conjugate formed/min/mg protein)</th>
<th>Superoxide dismutase (Units)</th>
<th>Glutathione reductase (µmoles of NADPH oxidized/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>8.45 ± 2.2</td>
<td>0.214 ± 0.05</td>
<td>13.7 ± 4</td>
<td>23.25 ± 2.5</td>
<td>25.3 ± 5.8</td>
</tr>
<tr>
<td>II</td>
<td>2.5 mg Mon/kg b.w.</td>
<td>5.47 ± 0.8**</td>
<td>0.172 ± 0.04</td>
<td>11.1 ± 2</td>
<td>10.04 ± 1.0***</td>
<td>18.6 ± 4.7**</td>
</tr>
<tr>
<td>III</td>
<td>5 mg Mon/kg b.w.</td>
<td>2.79 ± 0.7***</td>
<td>0.115 ± 0.02***</td>
<td>7.5 ± 1**</td>
<td>8.27 ± 2.1***</td>
<td>11.5 ± 2.0***</td>
</tr>
<tr>
<td>IV</td>
<td>10 mg Mon/kg b.w.</td>
<td>2.33 ± 0.7***</td>
<td>0.093 ± 0.01***</td>
<td>6.9 ± 2**</td>
<td>10.86 ± 3.3***</td>
<td>11.5 ± 3.2***</td>
</tr>
</tbody>
</table>

Values are the mean ± SD of 6 observations; ** p < 0.05, *** p < 0.01, **** p < 0.001 represent the comparison between the control and the treated group.

### Tab. 2. The effect of various doses of monensin on the levels of reduced glutathione and lipid peroxidation in the testis of Wistar rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Reduced glutathione (µmoles/mg protein)</th>
<th>Lipid peroxidation (nmoles of MDA formed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>0.908 ± 0.1</td>
<td>34.83 ± 5.3</td>
</tr>
<tr>
<td>II</td>
<td>2.5 mg Mon/kg</td>
<td>0.560 ± 0.2*</td>
<td>45.66 ± 7.2**</td>
</tr>
<tr>
<td>III</td>
<td>5 mg Mon/kg</td>
<td>0.269 ± 0.03***</td>
<td>54.83 ± 8.5***</td>
</tr>
<tr>
<td>IV</td>
<td>10 mg Mon/kg</td>
<td>0.282 ± 0.09***</td>
<td>60.1 ± 7.5***</td>
</tr>
</tbody>
</table>

Values are the mean ± SD of 6 observations; ** p < 0.05, *** p < 0.01, **** p < 0.001 represent the comparison between the control and the treated group.

Fig. 1. Agarose gel electrophoresis of genomic DNA isolated from testicular tissue treated with various doses of monensin for a period of 67 days. Lane 1 and 2: 10 mg monensin/kg; lane 3 and 4: 5 mg monensin/kg; lane 5 and 6: 2.5 mg monensin/kg; lane 7: control; lane 8: positive control.
the fragmentation of genomic DNA in all the treated groups (Fig. 1) as evidenced by distinct characteristic ladder formation.

Histological studies indicated marked degenerative changes in rat testis after monensin treatment (Fig. 2A–D). It was noticed that the degeneration of germ cells in the seminiferous tubules, decrease in number of various cell populations and formation of vacuoles had resulted from monensin treatment.

**Discussion**

The present study, demonstrated that monensin produced an antispermatogenic effect by inducing oxidative stress and DNA damage in rat testis. The decreased activity of catalase and glutathione peroxidase points towards the involvement of ROS in monensin’s action. In testis, H$_2$O$_2$ detoxification is shared by GSH-PX and catalase [30]. The inhibition in the activities of these enzymes possibly indicates that monensin may be increasing the generation of ROS levels in male germ cells. Oxidative damage has been implicated as a major factor in understanding of male infertility [1]. Monensin has been reported to increase the catalase activity in the liver and breast muscles of the broiler chickens when given at a concentration of 150 mg/kg of body weight while the activity of GSH-PX initially decreased and then tended to rise [28]. Administration of monensin also increased erythrocyte GSH-PX activity in broiler chickens [13]. Therefore, the induction of oxidative stress may be involved in the cellular effects of monensin which may be linked to the reproductive toxicity. Glutathione reductase (GR) main-
stains the cellular level of GSH by recycling oxidized glutathione, thus, playing an important role in the prevention of oxidative damage. The depletion in the activity of GR shows that the enzyme is actively involved in protecting the cells from the peroxidative damage of monensin. In the case of superoxide dismutase, a decreasing trend was noticed in almost all treated groups. SOD protects the cells from oxidative damage by removing the superoxide anion. The activity of glutathione-S-transferase was also found to be decreased with the drug treatment. The findings suggest that monensin is involved in ROS production in the male germ cells. Earlier, monensin has been shown to potentiate the oxidant production in pulmonary endothelial cells by increasing the intracellular pH [6]. It has been proposed that the Na⁺/H⁺ antiport activity of monensin is involved in regulating different cellular functions including apoptosis [34]. ROS have been implicated in the increased activity of the cellular Na⁺/H⁺ exchangers that is activated by phosphorylation in vascular myocytes from hypertensive rats [29].

A significant elevation in the levels of lipid peroxidation was observed along with the marked depletion of reduced glutathione levels. Monensin is known to dissipate the ion gradients in cell membranes and to alter their integrity, thereby leading to cellular dysfunctions including lipid peroxidation [21]. The increase in the lipid peroxide status of broiler chickens in acute monensin poisoning was also observed [28]. In the DNA gel electrophoretic study, it has been observed that monensin has caused fragmentation of DNA in all the treatment groups. Oxidative damage in testicular milieu is associated with DNA damage and may produce a higher frequency of abnormal sperms with significant deleterious effect on male fertility. An increase in cellular ROS production is often observed in apoptotic process triggered by various stimuli [12, 32, 33]. The induction of oxidative damage by the drug could be responsible for DNA damage. It has been demonstrated that the increased levels of lipid peroxides can produce DNA damage by inducing oxidative damage [16]. Previous studies have demonstrated that monensin can induce apoptosis in HeLa-60 cells by causing DNA fragmentation [34]. Histologically, marked degenerative changes in the seminiferous tubules were observed in monensin-treated animals, which may be due to the induction of oxidative stress and apoptosis. An elevated generation of ROS in the testis can lead to an alteration in tissue physiology [2]. ROS have also been involved in a number of pathophysiological conditions of testis [7]. From the obtained results, it may be concluded that monensin exhibits antispermatogenic effects by inducing the oxidative damage in rat testis as also substantiated by the histoarchitecture of the tissue. DNA fragments in all the monensin-treated groups further indicate that the pronounced antispermatogenic effect might have occurred at the level of the genomic DNA of the testis of the treated rats.

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References:

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