Histological and ultrastructural studies on the toxic effect of pan masala and its amelioration by *Elettaria cardamomum*

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**[ABSTRACT]**

AIM: To investigate the histological and ultrastructural changes observed in pan masala intoxicated mammalian testes under the effect of cardamom.

METHOD: Male Swiss mice were given pan masala orally at a dose of 2% of the feed and cardamom at a dose of 0.2% of the feed. They were divided into three groups, control (Group I), pan masala-treated (Group II), and a combination of pan masala and cardamom-treated group (Group III). Histologically, the testes of Group II mice displayed degeneration of tubular epithelium, disruption of spermatogenesis, and a marked reduction in germ cells.

RESULTS: When cardamom was given, damage was less with fewer distorted cells and also improvement with normal tubules and spermatid differentiation in Group III. Ultrastructurally, pan masala-treated testes showed cytoplasmic vacuolation, shrinkage and pyknotic nuclei of spermatogonia, and abnormal acrosomal granules.

CONCLUSION: When cardamom was given, the amelioration process was more evident showing a comparable morphology with control.

**[KEY WORDS]** *Elettaria cardamomum*; Pan masala; Testes; Histology; Ultrastructure

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**Introduction**

Pan masala is an extremely popular herbal blend sold throughout the Indian subcontinent. It is an assorted mixture of tobacco, catechu, areca nut, slaked lime, and certain food additives. Studies have reported that pan masala induced a reduction of sperm and sperm count, as well as daily sperm production after exposure to pan masala in a mouse model [1-2]. Further experimental studies also revealed testicular damage, abnormal morphology of sperm head shape, reproductive toxic potential, along with a significant decrease in acid phosphatase, alkaline phosphatase, and lactate dehydrogenase activity [3-4]. As a consequence of pan masala treatment in both male and female mice, a decline in reproductive performance and pregnancy outcome was observed. Cardamom, *Elettaria cardamomum* (L.) Maton (Zingiberaceae), is a plant native to the moist evergreen forests of the Western Ghats of southern India, and has been highly valued as a spice since ancient times. It is currently utilized in the medical field for the treatment of various types of diseases. The present study was designed to focus on the toxicological impact of pan masala on the testicular morphology of Swiss mice, and at the same time its response against cardamom treatment. Studies associated with biological and pharmacological markers against pan masala damage in the testes have been completed, where cardamom acted as an ameliorating agent. However, no work related to morphological assessment has been reported. Thus the present study focuses on the histological, ultrastructural, and morphometric parameters. Cardamom is used as an ameliorating agent, since spices contribute significantly towards an herbal cure against various disorders of the body. It is a well-known remedy for impotence and low sexual response. The therapeutic properties of cardamom oil are as an antiseptic, antispasmodic, carminative, cephalic, digestive, diuretic, expectorant, stimulant, stomachic, and tonic [5]. Linalool, one of the components of cardamom, has protective effects on the male reproductive system [6].

**Materials and Methods**

Fifteen male Swiss albino mice weighing (22 ± 5) g. acquired from B. N. Ghosh and Company, CIT Road, Kolkata, were housed in clear cages with wood chips as bedding and
were divided into three groups of five mice each. They were kept under controlled environmental conditions, including a temperature of (25 ± 5) °C and a 12 h light / dark cycle. The Group I was the normal control, Group II was fed with pan masala (2% of the feed), and Group III received a fixed combination of pan masala and cardamom, 2% and 0.2% of the feed, respectively for nine months. Various brands of pan masala were collected from the local market having almost similar compositions; the most popular brand was chosen for the present experiment. Cardamom was purchased from the local market. The investigation was cleared by the Ethical Committee, Ranchi University, Ranchi, for conducting research on Swiss mice and other strains of albino mice. The major components of pan masala are shown in Table 1.

### Table 1 Major components of pan masala

<table>
<thead>
<tr>
<th>Components of Pan masala</th>
<th>Major constituents</th>
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<tbody>
<tr>
<td>Areca nut</td>
<td>arecoline, arecaidine, arecolidine, guavacine and guvacoline</td>
</tr>
<tr>
<td>Catechu</td>
<td>tannins, polyphenols, 25%–35% catechu tannic acid, 2%–10% catechin</td>
</tr>
<tr>
<td>Lime</td>
<td>Ca(OH)₂</td>
</tr>
<tr>
<td>Tobacco</td>
<td>nicotine, tobacco-specific nitrates, N-nitro amino acids</td>
</tr>
<tr>
<td>Flavouring agent</td>
<td>menthol, musk etc.</td>
</tr>
<tr>
<td>Others</td>
<td>lead, cadmium, nickel</td>
</tr>
</tbody>
</table>

Cardamom is highly aromatic and distinctive spice. The percentages of the main components of volatile oil of cardamom are as follows: 1.5% α-pinene, 0.2% β-pinene, 2.8% sabinene, 1.6% myrcene, 0.2% α-phellandrene, 11.6% limonene, 36.3% 1, 8-cineole, 0.7% γ-terpinene, 0.5% terpinolene, 3% linalool, 2.5% linalyl acetate, 0.9% terpinene, 2.6% α-terpineol, 31.3% α-terpinyl acetate, 0.3% citronellol, 0.5% nerol, 0.5% geraniol, 0.2% methyl eugenol and 2.7% trans-nerolidol.

Animals were sacrificed after nine months by cervical dislocation under anesthesia, and testes were excised. A part of the excised testis was fixed in Bouin’s fixative (mixture of 75 mL saturated picric acid, 25 mL of 40% formaldehyde, and 5 mL of glacial acetic acid). The tissues were dehydrated through an ethanol series, then treated with xylene and embedded in paraffin wax. About 6 μm-thick sections of tissues were stained with hematoxylin and eosin (HE), and observed under the light microscope. Five tubules from five sections of each animal were taken for the analysis of the diameter of the seminiferous tubule and its quantification was done in both control and treated groups with the help of Image Tool software. Data were statistically analyzed using one way ANOVA (F > F crit was considered significant). The percentage of damaged tubules in the treated group was calculated using the following formula:

\[
\text{Percentage of damaged tubules} = \left( \frac{\text{Number of damaged tubules}}{\text{Total number of tubules}} \right) \times 100
\]

Another portion was fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol·L⁻¹ phosphate buffer for 8–12 h at 4 °C, and then processed to observe its ultrastructure by Philips CM-10 transmission electron microscope (Netherland) at AIIMS, New Delhi.

### Results

#### Histological study

Testes of control mice showed normal features of testicular tissue, namely seminiferous tubules and their epithelial cells, Sertoli cells and the germ cells at various stages, covering the complete process of spermatogenesis. There were three layers of cell, namely, the spermatogonia, primary and secondary spermatocytes; spermatoids and finally mature spermatozoa (Fig. 1, Ia and Ib). All sections of the testicular tissues obtained from the pan masala-treated Group II mice displayed several histopathological changes. The seminiferous tubules showed a loss and degeneration of the epithelium with infiltration of spermatogonial cells, necrotic spermatogonia and primary spermatocytes. Sertoli cell showed vacuolization and the spermatocytes and spermatids showed nuclear pyknosis and cytoplasmic vacuolization. There seemed to be no differentiation between the various layers of spermatogonial cells with accumulated sperms at the center of the tubule (Fig. 1, Ila and Iib). The germinal epithelium of the affected tubule appeared disorganized and disrupted, and showed shedding of germ cells into the lumina. There was a complete loss of elongated spermatids and spermatozoa, which means that spermatogenesis was arrested at the stage of rounded spermatid formation under the effect of pan masala treatment.

An amelioration process in Group III mice administered with cardamom and pan masala together showed less damage than the pan masala -reated group. Spermatids were visible in the lumen, which was not prominent in the section of Group II mice (Fig. 1, IIIa). There was a reduction in the number of abnormal tubules along with improvement in the spermatid differentiation (Fig. 1, IIIb).

However, some tubules showed necrosis with the presence of vacuoles inside. Detachment of Sertoli cells from the basement membrane and dilated intercellular spaces between germ cells with the appearance of vacuoles of atrophied Sertoli cells were also noticed.

Seminiferous tubular diameter showed a reduction in both the treated groups over a period of nine months; however, the damage seemed less in the Group III as compared to the Group II animals. Tubular degeneration was also more in Group II as compared to Group III, as shown in Table 2. The epididymis of the control group showed normal columnar epithelium and accumulated sperms in the lumen as shown in Fig. 2a. Sterocilia were easily visible which were actually long microvilli, which increase greatly the surface area of the columnar cells. When the mice were treated with pan masala for nine months, fusion and distortion of several tubules with sloughing of cells in the lumen was observed (Fig. 2b). It also showed the appearance of small to large vacuoles in the epithelium, and a decrease or absence of elongating spermatids (Fig. 2c).
Fig. 1 Photomicrography of each study group. Ia: testicular parenchyma of Group I mice after nine months (HE, × 40); Ib: testicular parenchyma of Group I mice after nine months. (HE, × 10); IIa: testicular parenchyma of Group II mice after nine months (HE, × 40); IIb: pan masala treated testes (Group II) for nine months displaying severe testicular damage. (HE, × 10); IIIa: testicular parenchyma of Group III mice after nine months (HE, × 40); IIIb: pan masala and cardamom treated testes (Group III) for nine months displaying almost normal tubules along with few damages. (HE, × 10)

Table 2 Diameter and % tubular damage of control and treated groups (mean ± SD, n = 5)

<table>
<thead>
<tr>
<th>No. of Mice</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Seminiferous tubule diameter /μm</td>
<td>% of damaged tubules</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>176.0 ± 0.85</td>
<td>169.4 ± 1.19*</td>
<td>169.8 ± 0.34*</td>
<td>-</td>
<td>73</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>177.2 ± 0.00</td>
<td>166.4 ± 0.93*</td>
<td>170.6 ± 0.23*</td>
<td>-</td>
<td>85</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>175.4 ± 1.27</td>
<td>170.2 ± 1.75*</td>
<td>172.2 ± 1.36*</td>
<td>-</td>
<td>78</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>177.4 ± 0.14</td>
<td>166.8 ± 0.65*</td>
<td>170.0 ± 0.20*</td>
<td>-</td>
<td>70</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>180.0 ± 1.98</td>
<td>165.8 ± 1.36*</td>
<td>168.8 ± 1.05*</td>
<td>-</td>
<td>82</td>
<td>45</td>
</tr>
</tbody>
</table>

Made at × 400 with sections from the right testis of five animals; *F > Fcrit vs. control

Fig. 2 Photomicrography of the epididymis of each group mice after nine months. a: Group I (HE, × 40); b: Group II showing destructed tubule (HE, × 40); c: Group II showing distorted and vacuolated tubular epithelium (HE, × 40); d: Group III showing normal epithelium with multinucleated sperm in the lumen (HE, × 40); e: Group III showing degenerating, as well as normal, epididymal tubes. (HE, × 40)
Ultrastructural study

The electron micrographs of the control mice testes showed the early spermatids with rounded cells and large spherical nuclei which contained chromatin clumps in a lightly stained cytoplasm; mitochondria at this stage tend to aggregate at the periphery of the plasma membrane with visible Golgi apparatus (Fig. 3a).

![Electron micrograph. a: the control testes after nine months (× 800, Scale: 2 µm); b: pan masala-treated testes after nine months (× 1450, Scale: 2 µm); c: pan masala and cardamom-treated testes after nine months (× 2 600, Scale: 1 µm)](image)

The third group which was fed with cardamom and pan masala together for nine months showed a comparable morphology with control. The epithelium was normal with various sperms in the lumen. They seemed normal with few multinucleated spermatid (Fig. 2d). Cell shrinkage and necrosis or pycnosis of the nuclei were also noticed (Fig. 2e). However, other tubule appeared normal.

Discussion

The testis is considered to be the most important organ in the male reproductive system. It is characterized by two main functions, synthesis of steroid hormones and production of spermatozoa[11]. Various factors could affect spermatogenesis, among these factors are chemical agents, such as medicines and toxic elements in the environment[12]. The results obtained in the present study, agree with those obtained by other authors[13] who reported that pan masala reduced testes weight in mice and enhanced the frequency of morphological abnormalities. It has also been reported that chronic feeding of pan masala affected the mouse germinal cells by inducing sperm-head abnormalities apart from impairing liver function in rats[14-15].

Post pan masala administration, spermatogonia demonstrated severe defects. They lost their normal shapes, possessing features of necrotic cells. As De Rooij and Russell[16] described, spermatogonia are particularly vulnerable to toxins and physical agents. Different defects in spermatocytes and spermatids post pan masala treatment may reflect the disturbances in the micro environment of the Sertoli cells that affect the protein synthesis machinery essential for germ cell differentiation. Proteins necessary for the differentiation of germ cells are secreted at their highest rates in the testes during spermatid elongation and spermiation[17]. The manifestations are clearly pathological and the affected cells become non-viable or dead and hence do not go through the process of spermatogenesis and/or spermiogenesis. Such cells are lost from any epithelium through either necrosis or apoptosis[18]. The seminiferous epithelium responds to such a situation with the premature release of the affected cells, which reach the epididymal duct for further processing and removal[19-20]. However, the damage was less in the cardamom-treated group. They showed normal spermatogonia with abundant spermatids in its lumen. The results of the present study clearly point to the germ cells present in the adluminal compartment of the seminiferous epithelium as the principal target of pan masala toxicity, and also its amelioration by cardamom in Swiss mice. Cardamom showed a promising effect against pan masala damage in the testes of Swiss mice. It possesses a large variety of bioactivities, such as antiulcer, antispasm, anticancer, anti-inflammatory, antioxidant, and analgesic[21-25]. However, the function and the related mechanism of action of cardamom on the male reproductive system are not well understood.

Conclusion

In summary, the present study indicated that cardamom showed protective effects against damage to the testicular tissue of male mice induced by pan masala. The present study also showed that pan masala administration in adult male mice exerts a clear effect on testicular structures and ultra-structures including degenerated changes of germ cells, and Sertoli cells. These changes reflect on their functions exerting deficiency in their performance. This condition was recovered in the mice treated with cardamom. Consequently, it can be said that his herb has promising effects against the toxic effect of pan masala and the same can be used in the medical
field to improve human health.

**Acknowledgement**

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**References**


[16] De Rooij DG, Russell LD. All you wanted to know about spermotogonia but were afraid to ask [J]. J Androl, 2000, 21 (6): 776-798.


