EFFECTS OF ATROPINE SULFATE ON RAT HARDERIAN GLANDS: CORRELATION BETWEEN MORPHOLOGICAL CHANGES AND PorphyrIN LEVELS

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ABSTRACT — We examined the correlation between histopathological changes and porphyrin levels in the Harderian glands of male rats after treatment with atropine sulfate.

After a single administration of atropine sulfate (250 mg/kg/day), the porphyrin levels in the Harderian glands gradually increased, beginning from 2 hr after administration, and at 36 hr reached a maximum level, which was about 7 times higher than that of the control animals. Histopathologically, the Harderian glands showed marked luminal dilatation and a brownish pigment accumulation in the lumina 6 hr after a single dose.

Under daily repeated administrations of atropine sulfate (250 mg/kg/day), the highest porphyrin levels in the Harderian glands were observed 24 hr after the third dose, and were about 9 times higher than those of the control animals. However, beginning from one week after the initial dose, much lower peak porphyrin levels were observed 6 hr after each dose. The maximum porphyrin levels were only twice as high as those of the control animals, and they returned to the control levels 24 hr after each atropine dose. Histological examinations of the Harderian glands revealed that repeated administrations of atropine sulfate induced the same histopathological changes observed after a single atropine administration, and that no aggravated dilation of the lumina or pigmentation in the lumina appeared after such repeated administrations. The degree and incidence of the histopathological changes observed correlated well with the porphyrin levels. Some animals showed a degeneration of the glandular epithelium after 4 weeks of treatment, and the frequency increased slightly after 13 weeks of treatment.

The present study suggests that atropine suppresses the expulsion of secretory materials, including porphyrin, from the glandular lumen of the Harderian glands, and thereafter an excessive accumulation of porphyrin induces luminal dilatation. These changes were gradually reduced by repeated administrations. The degeneration of the glandular epithelium after repeated administration might be a consequence of retention of an excessive accumulation of porphyrin.

KEY WORDS: Harderian gland, Porphyrin, Histopathological changes, Atropine sulfate, Rat

INTRODUCTION

The Harderian gland is a tubuloalveolar gland located in the eye orbits in rodents such as rats, mice and hamsters (Sakai, 1981). Its functions have not been clarified completely, although the Harderian glands are thought to be a source of lubrication for the eyes (Cohn, 1955, Kennedy, 1970), a source of pheromones.
(Thiessen et al., 1976; Payne, 1979), involved in modulation of reproductive function (Clabough and Norvell, 1973, 1974), thermoregulation (Thiessen and Kittrell, 1980), and as a site of immune response (Mueller et al., 1971, Albini et al., 1974, Burns, 1979). One of the characteristics of the glands in rodents is the presence of lipids and porphyrin in the lumina. Porphyrin is a pigment which develops a red-colored fluorescence when exposed to ultra-violet light (Derrien and Turchini, 1924). Shirama et al. (1980) reported that the concentration of porphyrin in the Harderian gland changed due to the estrus cycle (lower in metestrus and higher in diestrus). We have previously reported that atropine sulfate, an antimuscarinic agent, induced morphological changes in the Harderian gland, including dilation of the lumina and accretions of brownish pigments in the glands. In addition to these alterations, degeneration of the glandular epithelium was also prominent in prolonged treatments in atropine sulfate (Hayasaka et al., 1992).

As Harderian glands in rats have been known to discharge porphyrin, we hypothesized that atropine-induced dilatation of the lumina would cause an excessive accumulation of porphyrin in the lumina. In the present studies, we examined porphyrin levels in the histopathological features of rat Harderian glands to clarify the pathogenesis of morphological changes in the lumina treated with atropine sulfate.

MATERIALS AND METHODS

Animals
Five-week-old Slc:SD male rats were purchased from Shizuoka Laboratory Animal Center, Japan, and quarantined and acclimatized for a week. All animals were housed in stainless steel cages in rooms controlled for temperature (23 ± 2°C), relative humidity (55 ± 10%), light-dark periods (7:00-19:00) and fresh air changes (10-35 times/hr). They were allowed free access to pelleted food (CRF-1, Oriental Yeast Co., Ltd.) and tap water. They were divided into experimental groups consisting of 5 rats each.

Chemicals
Atropine sulfate of a technical grade with a purity of 97.1% was obtained from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. It was dissolved in distilled water to produce a 2.5% w/v solution, and was administered orally at 250 mg/kg once daily at a standard volume of 10 mL/kg/day.

Experimental design
1. Single administration
Rats were euthanized by exsanguination under ether anesthesia at 2, 6, 12, 18, 24, 30, 36, 42 and 48 hr after administration. Both Harderian glands were removed. One gland was weighed and stored at −70°C to be used for porphyrin analysis, and another was subjected to light microscopic examination. Non-treated rats were euthanized by the same method at 0, 6, 12 and 18 hr, and their organs were removed and examined in the same manner.

2. 12-day administration
Rats were euthanized by exsanguination under ether anesthesia at 2, 6, 12, 18 and 24 hr after the final dose in a series of 12-day consecutive daily administrations. Both Harderian glands were removed and processed by the above-mentioned method.

3. 13-week administration
Rats were euthanized by exsanguination under ether anesthesia at 6 and 24 hr after 1, 3, 7, 14, 28 or 91 days of administrations. Both Harderian glands were removed and processed by the above-mentioned method. Control rats were euthanized 24 hr after each period.

Measurement of Harderian gland porphyrin content

Five rats were euthanized at each point, and their Harderian glands were removed and weighed according to the expected porphyrin concentration. For assay, they were thawed and homogenized with a small amount of methanol. To esterify the porphyrin, the homogenate samples were placed in 10% sulfuric acid in methanol, shaken occasionally, and left to stand for 24 hr at 37°C in the dark. After cooling to 20°C, the solution was filtered. The clear filtrate was used for the determination of total porphyrin methyl ester using a spectrofluorimeter (Hitachi-204) with an excitation wavelength of 407 nm and an emission wavelength of 605 nm. Dimethyl-protoporphyrin was used as a reference standard, since it is thought that the main porphyrin in the Harderian gland was protoporphyrin (Sanitrak and Krijt, 1987; Park et al., 1996; Kennedy, 1970; Hugo et al., 1987).

Light microscopic examination
The Harderian glands were removed and fixed in
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10% neutral buffered formalin solution. They were examined histologically after routine processing for embedding in paraffin and staining hematoxylin-eosin.

The areas occupied by brown pigments in the lumina were planimetrically assessed on light micrographs (photographed with an Olympus-Avio SP-5000, Japan) at ×50 magnifications by means of a digitizer.

Statistical analysis

The porphyrin contents in the Harderian glands of the control and treatment groups were calculated and compared using Student’s t-test. A p value less than 0.05 was regarded as significant.

RESULTS

Porphyrid levels in the rat Harderian glands

1. Single administration

Porphyrin levels in the Harderian glands of the rats following a single administration of atropine are shown in Fig. 1. The porphyrin levels in the glands of the control rats ranged from 295 to 406 µg/g gland tissue. After a single administration of atropine sulfate, the porphyrin levels increased significantly, starting from 2 hr after administration, and reached a peak of 2613 µg/g at 36 hr, which was 7 times higher than that of the control group. Thereafter the porphyrin levels decreased, and the level was 593 µg/g at 48 hr, which was slightly higher than that of the control group.

2. 12-day administration

Fig. 2 shows porphyrin levels in the Harderian glands of the rats treated with atropine sulfate for 12 days. The porphyrin levels continued to increase until 6 hr after the final dose, following a pattern similar to that observed in the rats administered a single dose of atropine sulfate. Thereafter, however, the porphyrin levels showed a slight increase, and a peak level of 1039 µg/g was observed at 12 hr. The porphyrin level at 24 hr was 311 µg/g, which was similar to the level of the control group (Fig. 1). In comparison with single administration, 12-day administration showed a slight increase of porphyrin levels and a shorter time course to recovery.

3. 13-week administration

Changes in porphyrin levels 6 and 24 hr after the last dose from day 1 to 13 weeks are shown in Fig. 3. The porphyrin levels in the control animals were distributed between 300 and 400 µg/g, indicating no apparent fluctuation in those levels in the control animals throughout the experimental period. In the animals treated with atropine sulfate, the porphyrin level 24 hr after a single treatment was 2109 µg/g. The level at 24 hr after 3-day administration reached 3731 µg/g, which was more than 9 times higher than that in the controls. However, the porphyrin level decreased to about 400 µg/g 24 hr after the seventh dose, which was comparable to that of the control group. Such decreased porphyrin levels were maintained for the rest of

Fig. 1. Porphyrin concentration in the Harderian gland of rats following a single oral administration of atropine sulfate.
Each value represents mean ± S.D. (n=5).
Significantly different from the control value.

Fig. 2. Porphyrin concentration in the Harderian gland of rats following a 12-day oral administration of atropine sulfate.
Each value represents mean ± S.D. (n=5).
The data of a single treatment show the result of examination 1.
the experimental period. In contrast, the porphyrin levels 6 hr after each dose were constant, and usually 2 times higher than those of the control group throughout the experimental period.

**Histopathological Findings in the Harderian Glands**

1. **Single administration**

Results of the histopathological examination of the Harderian glands of the rats after a single atropine sulfate administration are shown in Table 1. Small amounts of brownish pigment accumulation in the alveolar lumina and acinar dilatation were observed in the Harderian glands of 1 out of 5 control animals. No significant changes were observed in the Harderian glands of the atropine sulfate-treated rats at 2 hr after single administration. However, an increase of brownish pigment secretion in the lumina and dilatation of the lumina were observed at 6 hr, and thereafter the changes became severe, reaching a maximum at 36 hr. Although the degree and incidence of these changes decreased, they had not completely recovered to the control levels even at 48 hr after a single administration of atropine sulfate.

2. **12-day administration**

Table 2 shows the results of the histopathological examination of the Harderian glands of the rats administered atropine sulfate for 12 days. At 2 hr after the final dose, 4 out of 5 animals showed dilatation and brownish secretions in the lumina. The extent and incidence of these findings reached a maximum at 6 and/or 12 hr after the 12-day administration, and thereafter began to ameliorate.

3. **13-week administration**

Results of the histopathological changes in the Harderian glands at 6 and 24 hr after the final administration of atropine for 1, 3, 7, 14, 28 or 91 days are shown in Table 3. The sequential changes in dilatation of the lumina and the degeneration of the glandular epithelium in the Harderian gland are shown in Fig. 4 and Fig. 5, respectively.

In the control animals, the dilatation of the lumina was observed in about 20% of the animals during the experimental period. In the atropine sulfate-treated animals, dilatation of the lumina was observed in all of the animals 24 hr after the first and third doses (Fig. 4, Photo 1). However, the incidence of this histopathological change decreased gradually, and recovered to a level comparable to the control after 28 days. On the other hand, dilatation of the lumina was observed in no less than 80% of the animals 6 hr after each dose from days

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**Table 1.** Histopathological findings in the Harderian gland of rats after a single oral administration of atropine sulfate.

<table>
<thead>
<tr>
<th>Findings</th>
<th>Control</th>
<th>Hr after a single administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade</td>
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</tr>
<tr>
<td>Dilatation of lumina</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1</td>
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<tr>
<td></td>
<td>++</td>
<td>0</td>
</tr>
<tr>
<td>Brown secretion in lumina</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1</td>
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<td></td>
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</table>

- negative, + mild, ++ moderate.
Numbers represent the No. of rats that showed the findings.
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**Fig. 4.** Sequential changes in dilatation of lumina in the Harderian gland of rats treated orally with atropine sulfate. (n=4-5)

**Fig. 5.** Sequential changes in degeneration of glandular epithelium in the Harderian gland of rats treated orally with atropine sulfate. (Number of control rats is 5, and number of atropine sulfate is 9-10)

**Table 2.** Histopathological findings in the Harderian gland of rats treated orally with atropine sulfate for 12 days.

<table>
<thead>
<tr>
<th>Findings</th>
<th>Grade</th>
<th>2hr</th>
<th>6hr</th>
<th>12hr</th>
<th>18hr</th>
<th>24hr</th>
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<tbody>
<tr>
<td>Dilatation of lumina</td>
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<td>3</td>
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<tr>
<td>Brown secretion in lumina</td>
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<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
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</tbody>
</table>

- negative, + mild, ++ moderate
Numbers represent the No. of rats that showed the findings.

**Table 3.** Histopathological findings in the Harderian gland of rats treated orally with atropine sulfate for 91 days.

<table>
<thead>
<tr>
<th>Treatment period</th>
<th>1 day</th>
<th>3 days</th>
<th>7 days</th>
<th>14 days</th>
<th>28 days</th>
<th>91 days</th>
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<tr>
<td>Findings</td>
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<td>6hr</td>
<td>24hr</td>
<td>6hr</td>
<td>24hr</td>
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<tr>
<td>Dilatation of lumina</td>
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<td>4</td>
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<tr>
<td>Brown secretion in lumina</td>
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<td>1</td>
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<td>5</td>
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<tr>
<td>Degeneration of glandular epithelium</td>
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<td></td>
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<tr>
<td>+</td>
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C: control group, T: treatment group, 6hr: 6 hr after the final treatment, 24hr: 24 hr after the final treatment,
- negative, + mild, ++ moderate
Numbers represent the No. of rats that showed the findings.
Photo 1. The Harderian gland from the rat treated orally with atropine sulfate for 3 days. Dilatation of the lumina and brown pigments in the lumina of the Harderian gland are seen. H. E. staining, ×35.

Photo 2. The Harderian gland from the rat treated orally with atropine sulfate for 28 days. Minimal degeneration of glandular epithelium and brown pigments in the lumina are seen. H. E. staining, ×70.
1 to 14, and was still observed in 60% even on day 91. As to changes in the glandular epithelium, degeneration was observed in 20% and 40% at 28 and 91 days, respectively (Fig. 5, Photo 2). This increase was very slight and was not enhanced by the prolongation of the atropine treatments, even though it seemed that the incidence of degeneration had increased. The relationship between the porphyrin level and the histopathological changes in the luminal area in the Harderian gland of rats treated with atropine sulfate is shown in Fig. 6. A clear correlation was found between the porphyrin level and the area of the lumina ($r=0.887$).

**DISCUSSION**

We previously reported that atropine, an antimuscarinic drug, caused luminal dilatation and formation of brownish pigment droplets in the Harderian gland of rats, and eventually induced degeneration of the glandular epithelium over a prolonged treatment period (Hayasaka et al., 1992). As the Harderian gland was supplied by cholinergic nerves, Tashiro et al. (1940) and M’azl’o and Rohonyi (1963) have reported that injection of cholinomimetic substances leads to chromodacryorrhea (secretion of bloody tears containing porphyrin pigments) from the Harderian gland in rats. To investigate the possible causes of atropine-induced morphological changes, including luminal dilatation, we designed an experiment to determine the role of porphyrin accumulation.

Porphyrin content in the Harderian gland increased significantly to 495 μg/g 2 hr after an administration, in contrast with the control. The levels peaked at 36 hr (2613 μg/g), and were more than 7 times those of the controls. Afterwards the levels decreased, but the content had not recovered to levels comparable to the controls by the 48-hr point. In the present study, the area of luminal dilatation was measured after atropine dosing. Histopathologically, the degree of the lumina and extent of the brownish pigments in the lumina correlated well with the porphyrin levels. The apparent correlation between the porphyrin content and the area of the lumina ($r=0.887$) is of particular interest. These findings indicate that atropine-induced luminal dilatation in the Harderian gland is due to an excessive accumulation of porphyrin in the lumina.

Satoh et al. (1992) reported that rat Harderian glands exhibited a tubuloalveolar structure with relatively wide lumina, in which some osmiophilic dense droplets exocytosed from the glandular cells were observed. Two types of glandular cells (type A and type B), sometimes showing exocytotic figures of lipid-secretory vacuoles, were recognized, as well as myoepithelial cells. Moreover, after injections of carbamylcholine chloride or bethanechol chloride, which have a muscarinic action, many of the alveolar lumina were dilated and empty, although some lumina contained a considerable number of osmiophilic droplets. Enhanced exocytosis and a pronounced decrease in the number of vacuoles in the glandular cells were observed. However, these changes were inhibited to almost negligible levels by atropine. They reported that the injection of carbamylcholine chloride causes amplified exocytosis of the glandular cells and contraction of the myoepithelial cells in rat Harderian glands. They considered that cholinergic systems regulated the secretion of rat Harderian gland cells which have muscarinic receptors. Harkness reported that chromodacryorrhea was produced in rats following the injection of acetylcholine, but that these changes were inhibited by atropine (Harkness and Ridgway, 1980). Chiquoine (1958) thought that acetylcholine caused the release of red tears, probably through myoepithelial cell contraction and expulsion of the secretion from the glandular lumina. As mentioned above, the increase in porphyrin contents and brownish pigment droplets appears to suggest that atropine probably inhibits the release of secretory materials in the Harderian gland by an antimuscarinic effect, and leads to depositions of excessive secretory materials in the lumen. It can be further postulated that dilatation of the glandular acinus induced by atropine may be caused by a mechanical action; excessive secretory materials are retained in the lumina for a long period, and then physically induce dilatation of the glandular acinus.

The dilatation of the lumina and the porphyrin lev-
els in the Harderian glands of rats following repeated administrations of atropine were lower than the levels from a single administration. Therefore, sequential changes in both the dilatation and porphyrin levels following 12 repeated administrations were examined. Those changes were the same as those following a single-treatment of atropine until about 6 hr, but their levels at 12 hr or more did not increase as much as those of a single administration. These results suggested that the animals became tolerant of pharmacodynamic effects as a result of repeated atropine administrations, and that the changes contributed to the decreased duration of the response to a given dose of atropine.

The repeated administration of atropine induced a degeneration of the glandular epithelium after 4 weeks, and after 13 weeks the frequency of degeneration reached as high as approximately 40%. This change may be due to an excessive accumulation of porphyrin in the lumina, with acinal cells then being pressed secondarily by this porphyrin. The alterations in the epithelium may be due to the secondary effect of the retention of excessive secretory materials.

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