RESEARCH ARTICLE

Effect of Perchlorate on Ovarian Morphology and Histology, Estrous Cyclicity, Steroidogenic enzyme activities and hormonal profiles in Adult Female Rats

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Abstract

Perchlorate contamination is widespread for its industrial uses and natural occurrence. It is also used as an antithyroid drug. Perchlorate competitively inhibits iodide transport at the level of sodium-iodide symporter (NIS) in the thyroid gland, affecting thyroid hormone synthesis, resulting in alteration of thyroid gland structure and functions. Thyroid hormones are vital for proper functioning of the female reproductive system since they modulate the metabolism and development of the ovary and uterus. This study investigates the effect of perchlorate exposure for four consecutive weeks on the morphological and functional status of reproductive organs in adult female rats following parameters such as the estrous cyclicity, histology of ovary and uterus, and activities of steroidogenic enzymes, serum estradiol and estriol levels. Results revel irregular estrous cyclicity that ultimately ceased presenting a persistent estrous stage, hypertrophied ovary with corpora lutea of different sizes, large and inflated uterine lumen with proliferated endometrium comprising numerous secretory glands in the perchlorate exposed reproductive organs. The activities of steroidogenic enzymes (Δ^5 3β HSD and 17β HSD) were augmented that have heightened serum estriol level while the estradiol level was weakened. Therefore, an apparent hypertrophied and hyperfunctioning condition of ovary and uterus developed resembling pseudopregnancy on exposure of this iodide blocker.

Keywords: Female reproduction, Iodide blocker, Ovary, Perchlorate, Steroidogenic enzymes

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Introduction

Iodine is an indispensable component of thyroid hormones, triiodothyronine (T3) and thyroxine (T4). Normal thyroid function is dependent on an adequate supply of iodine. Most of the ingested iodine is accumulated in the thyroid gland and is available for the biosynthesis of the hormones.[1] Iodine accumulation in the thyroid is an active transport process, occurring against the iodide electrochemical gradient, stimulated by TSH (thyroid stimulating hormone) through the sodium-iodide (Na+/I-) symporter (NIS).[2-6] NIS is an intrinsic plasma membrane transport protein found on the basolateral membrane of thyroid cells coupling the inward downhill translocation of Na+ with the inward uphill translocation of iodide (I-). The activity of NIS is Na+ dependent, electrogenic and the stoichiometry of cotransport is $2Na^+$: 1 anion.[7] The well-known classic competitive inhibitors, anions like thiocyanate block iodide transport into thyroid follicles by blocking NIS.[1]

Perchlorate (ClO^{4-}) is another such anion that competitively inhibits iodide at the level of NIS with 30 times the affinity for iodide.[8] The active transport of perchlorate by NIS is in a dose-dependent manner in the thyroid.[9,10] The inhibition is precisely on account of the steric hindrance of the bulky perchlorate ion as it resembles iodide sterically. Its mode of action is to compete with iodide for the symporter. Perchlorate is a more potent inhibitor than thiocyanate. Perchlorate is also able to discharge intrathyroidal iodide rapidly as it gets accumulated itself in the thyroid follicular cells.[11] Thus, perchlorate inhibits iodine uptake by the thyroid gland and subsequently decreases thyroid hormone production.[12] These properties of perchlorate have been exploited since the early 1950s for clinical purposes, like treatment of severe cases of amiodarone- or iodine- induced thyrotoxicosis and short-term inhibition of thyroidal radioactive iodide accumulation after administration of contrast media for angiographic studies and computed axial tomography.1

In the present scenario, the availability of perchlorate in the surface and groundwater sources found abundant on account of its natural occurrence[13,14] as well as industrial production well-off. It is also used as an oxidizer in solid rocket propellant, slurry explosives, road flares and airbag inflation systems.[15] Low level of perchlorate exposure appears to be ubiquitous. It has also been detected in the drinking water of India, although the level as found was lower than

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that observed in U.S.[16] Perchlorate is very stable and readily forms salt with cations like magnesium, potassium, sodium, ammonium etc.[17] Earlier studies suggest that the action of perchlorate is profound on thyroid gland besides its adverse effects on other systems. However, the available data indicates its effect on female reproduction has not been studied adequately. Thyroid hormones have been suggested to act intricately the reproductive functions. Therefore, there might have the effect of perchlorate exposure on the structure and functions of the female reproductive system as well as the ovarian steroidogenesis. Hence, the present investigation has been undertaken to evaluate the effects of perchlorate on adult virgin female rats by exposing them orally with a non-toxic dose of perchlorate solution. In this context ovarian morphology and histology, estrous cyclicity, steroidogenic enzyme activities and hormonal profiles in adult female rats were studied.

MATERIALS AND METHODS

Animal Maintenance: For the investigation, twelve adult female virgin albino rats of Wistar strain, weighing 110 ± 10 gm were used. Animals were maintained as per national guidelines and protocols, approved by the Institutional Animal Ethics Committee (IAEC). They were housed in clean polypropylene cages in two divisions, having six each in a well-maintained environment of temperature 22 ± 2 °C and humidity 40-60% in animal house under twelve-hours light/twelve-hour dark cycles. Animals were fed on a standardized normal diet and water ad libitum.[18]

Dose of Perchlorate: Potassium salt of perchlorate (Loba, CAS: 7778-7-4-7) was prepared by dissolving it in double distilled water. 46.4 mg/kg body weight of this solution was administered daily by oral route through force-feeding to each rat.[19]

Study of Estrous cycle: Normal saline (0.9 gm%) was taken in a blunt-tipped dropper and was pushed into the vagina of each rat of both the treated as well as the control groups of rats.[20] The fluid from the vagina was sucked out and taken on a clean slide. The smear was spread uniformly, dried, fixed by dipping in methanol and then stained by hematoxylin and eosin (H & E). The slide was then observed under a light microscope to identify the stage of the estrous cycle. The entire protocol was performed for 28 consecutive days of study and the data was recorded.

The sacrifice of the animal: The animals were sacrificed on the consecutive day of the last treatment following institutional ethical procedure. Blood was collected separately from the hepatic portal vein of each animal. Before sacrifice, the body weight of each rat was taken.

Histological study: On the day of the sacrifice of the animals, the ovary and uterus of both the groups of animals were removed and weighed, fixed in Bouin's solution and embedded in paraffin. Sections of 5µ thickness were taken from the mid portion of the tissues and stained in hematoxylin and eosin for observation under light microscope.

Assay of Δ^5 3β HSD[21] and 17β HSD activity,[22] – The tissue was homogenized, maintaining a temperature of 4°C, with homogenizing fluid containing 20% spectroscopic grade glycerol, 5 mM potassium phosphate and 1 mM EDTA at a tissue concentration of 100 mg/mL homogenizing mixture in Potter-Elvehjem glass homogenizer and centrifuged at 10000 g for 30 min in a cold ultracentrifuge (REMI, C-30) at a constant temperature of 4°C. Then 200 µL of the supernatant was mixed with 1 mL of 100 µM sodium pyrophosphate buffer (pH 8.9) and 20 µL of 30 µg 17β-Estradiol. Δ^5-3β-HSD (Hydroxy Steroid Dehydrogenase) activity[21] was measured after the addition of 5 µM NAD (Nicotinamide adenine dinucleotide phosphate) to the cuvette in a UV spectrophotometer (UV-1240 Shimadzu, Japan) at 340 nm against a blank without NAD. One unit of enzyme activity is equivalent to a change in absorbance of 0.001/min at 340 nm. For measurement of 17β HSD enzyme activity,[22] supernatant was first prepared as described above. 200 µL of this supernatant was added with 1.5 mL of 440 µM sodium pyrophosphate buffer (pH 8.9), 0.5 ml bovine serum albumin and 40 µL of 0.3 µM 17β-Estradiol. 17β-HSD activity was measured after adding 1-mL of 1.35 µM NAD to the cuvette as described above.

Protein Estimation[23] – Proteins were estimated biochemically following the method of Lowry using bovine serum albumin (BSA) as the standard protein.

Enzyme-Linked Immunosorbent Assay of Serum E_2 and E_3 level – Serum Estradiol (E_2) and Estriol (E_3) were assayed using ELISA kits obtained from Dia Metra, S. R. L. Italy (Code No.DKO003 and DKO007 respectively). The sensitivity of the E_2 and E_3 assay were 8.7 pg/mL and 0.03 ng/mL, respectively.

Statistical Analysis[24] – Results were expressed as mean ± standard error (S.E). Significance of difference between the control and treated groups were estimated using student's two tail t test. p values < 0.05 were considered as statistically significant.

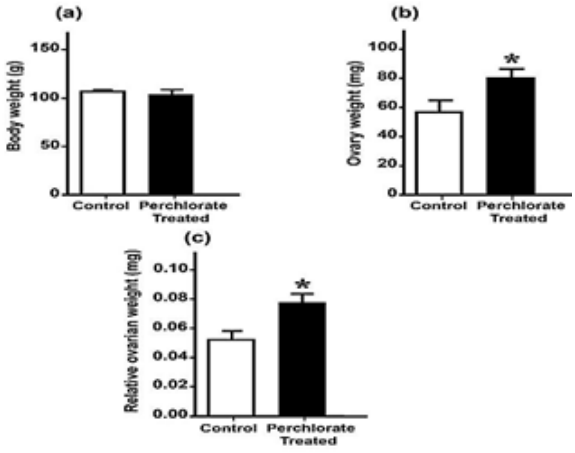

Figure 1: Effect of perchlorate on (a) body weight, (b) ovary weight and (c) relative ovary weight. Results are expressed as mean ± SE, n=6, *p < 0.05 as compared to control.

Table 1: Day-to-day recording of the stages of estrous cycles in the control and perchlorate treated animals for 28 days

Day rat	C1	C2	C3	C4	C5	C6	T1	T2	T3	T4	T5	T6
1	D	M	E	E	P	D	E	D	P	P	M	D
2	D	D	E	E	E	P	M	D	E	E	D	P
3	P	D	M	M	E	E	D	P	E	E	D	E
4	E	D	D	D	M	E	D	E	M	D	P	E
5	E	P	D	D	D	M	P	E	D	D	E	M
6	M	E	D	P	D	D	E	M	D	D	E	D
7	D	E	P	E	D	D	E	D	D	P	M	D
8	D	M	E	E	E	P	D	D	E	E	D	D
9	E	D	E	D	E	E	D	E	E	E	D	P
10	E	D	D	D	M	E	D	E	D	D	E	E
11	D	D	D	P	D	D	P	D	D	D	E	E
12	D	E	D	E	D	D	E	E	D	D	E	E
13	D	E	E	E	D	P	M	E	D	P	M	M
14	P	D	E	M	P	E	D	D	P	E	D	D
15	E	D	M	D	E	E	D	D	E	E	E	D
16	E	D	D	D	E	M	D	D	E	E	E	D
17	M	P	D	E	M	D	D	D	M	M	E	P
18	D	E	P	E	D	D	P	D	D	D	E	E
19	D	E	E	M	D	D	E	E	D	D	D	E
20	E	D	E	D	E	P	E	E	E	E	D	E
21	E	D	D	D	E	E	E	E	E	E	E	D
22	D	P	D	E	D	E	E	E	E	E	E	E
23	D	E	D	E	D	M	E	M	E	E	E	E
24	D	E	E	D	D	D	E	D	E	E	E	E
25	D	M	E	D	D	D	E	E	E	E	E	E
26	P	D	D	D	P	D	E	E	E	E	E	E
27	E	D	D	P	E	P	E	E	E	E	E	E
28	E	D	D	E	E	E	E	E	E	E	E	E

P = Proestrous E = Estrous M = Metestrous D = Diestrous.
$C_1 – C_{6:}$ Control group; $T_1 – T_{6:}$ Perchlorate treated group

Results

Effect of perchlorate on the weight of body, ovary and relative ovary weight in female rats – Perchlorate did not show any significant effect ($p > 0.05$) on the body weight, but as far as ovarian weight and relative ovarian weight concern, perchlorate exposure caused a significant increase ($p < 0.05$) in both the weights compared to respective control (Figure 1).

Effect of perchlorate on serum hormonal profiles in female rats – Perchlorate exposure caused a significant increase in the serum estriol (E_3) level but the serum estradiol (E_2) level was found to be decreased significantly ($p < 0.05$) as compared to respective control animals. The present study also demonstrated a significant increase ($p < 0.05$) in the ovarian delta-5, 3 beta hydroxy steroid dehydrogenase ($\Delta^5$3β HSD) and 17-beta hydroxy steroid dehydrogenase (17β HSD) activity as compared with respective controlcontrol (Figure 2).

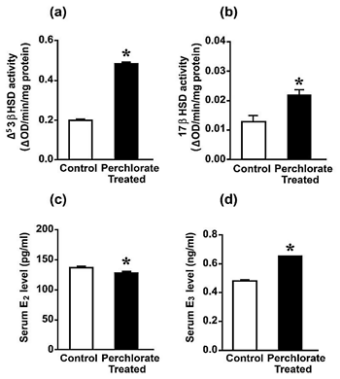

Figure 2: Effect of perchlorate on (a) $\Delta^5$3β HSD enzyme activity and (b) 17β HSD enzyme activity; (c) serum E_2 and serum (d) E_3 levels. Results are expressed as mean ± SE, n=6, *$p < 0.05$ as compared to control

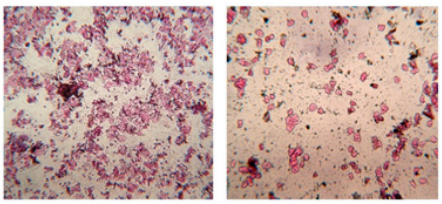

Figure 3: Photomicrograph of vaginal smear of estrous phase of estrous cycle of control and perchlorate treated animals. H & E x 40.

Effect of perchlorate on estrous cycle – The estrous cyclicity of both the control and the perchlorate exposed animals were studied for the entire period of experimentation i.e., for four weeks (28 days) and properly documented (Table 1). The control animals showed a normal estrous cyclicity of 98-106 hours in an average for the entire period of study whereas the estrous cycle of the perchlorate treated rats showed normal cyclical changes in the animals till 22 ± 2 days followed by an irregular cyclic stage with persistent estrous phase (Figure 3).

Histopathological studies – The histological section of ovary of perchlorate exposed rats showed the presence of numerous corpus luteum and absence of Graafian follicles compared to respective control rats where numerous antral and pre-antral follicles, along with Graafian follicles containing centrally located ovum were found present (Figure 4a).

The histological section of the experimental and control uterus was compared. The endometrial structure of the control rats showed a normal appearance having lumen at the center, surrounded by numerous glands, thickened endometrium and myometrium. However, perchlorate

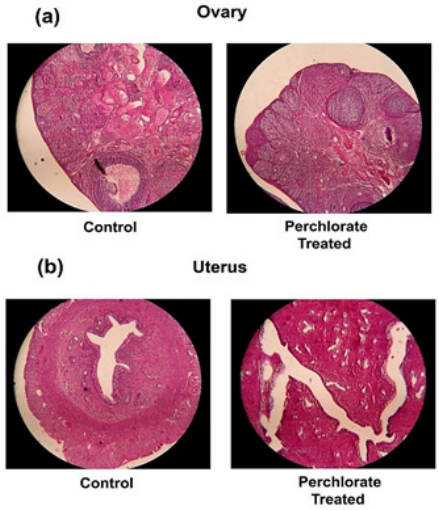

Figure 4: Photomicrograph of (a) Section of estrous phase control ovary (left panel) showing numerous antral and pre-antral follicles and Graafian follicle with ovum and estrous phase perchlorate exposed ovary (right panel) showing numerous corpus luteum without Graafian follicle;
(b): Section of estrous phase control uterus (left panel) showing complete appearance with lumen at centre surrounded by numerous glands, thickened endometrium and myometrium and estrous phase perchlorate exposed uterus (right panel) showing enlarged lumen along with its branches secretory glands. H & E x 40.

treatment caused structural disorientation of the lumen. There was an absolute loss of cellular architecture as evident by the constricted endometrium, myometrium and perimetrium (Figure 4b); the lumen of the uterus of the perchlorate treated animals was found distended with numerous glands.

Discussion

Perchlorate in pharmacological doses is widely used in the treatment of hyperthyroidism, as in thyrotoxicosis, Grave's Disease, etc.[12] In normal animals, prolonged perchlorate exposure in pharmacological doses may causes hypothyroidism as observed recently in our laboratory. Hypothyroidism affects the reproductive system in women more than in men. In rats, fetal hypothyroidism results in small ovaries.[25] Recent studies have reported that perchlorate potentially caused DNA damage in testicular cells and reduced testicular spermatogenic ability, resulting in reproductive toxicity in Wistar rats.[26] It has also been found to alter the morphology and biochemistry of sperms and sperm motility in Silurana tropicalis frogs.[27]

Earlier studies have demonstrated that perchlorate has no effect on body weight gain in the treated animals. The present study also showed no significant changes in body weight gain following perchlorate exposure compared to the control group and thus the observation is consistent with earlier findings. However, an increase in the ovarian weight, as well as its relative weight, has been observed after perchlorate treatment as compared to that of the control group. Therefore, perchlorate causes an increase in ovarian weight. On the contrary, ovaries are relatively small in PTU-induced rats or in thyroidectomized animals.[28] The exact mechanism by which perchlorate augments ovarian weight is yet to be elucidated.

The hormonal profile, namely estradiol and estriol, were assayed in the serum of the perchlorate exposed animals and found that the concentration of estradiol was decreased significantly but the concentration of estriol was increased as compared to the control. Available literature shows that during estrous stage of the estrous cycle of the control animals, estradiol level in serum is higher which causes maximum proliferation of the endometrial wall resulting in the production of cornified epithelial cells as observed in vaginal smear. In the present investigation, the estradiol level was decreased, but the estriol level was increased, which perhaps was found responsible for the proliferation and development of the endometrium resulting in the formation of more cornified cells. Therefore, the estrous stage as observed in perchlorate exposed animals, does not reflect true estrous but is for the development and maintenance of endometrium as found in pseudo-pregnant rat. To study the cause of enhanced estradiol production, the activity of the regulatory enzymes of steroidogenic pathway namely $\Delta^5$3β HSD and 17β HSD activity was measured in the ovary and found that the activity of these steroidogenic enzymes is much more than that of the control animals in estrous stage. Therefore, in perchlorate exposed animals, the enhanced estriol production is for the increased activities of these steroidogenic enzymes. It may be mentioned that estriol is not the direct product of steroidogenic pathway and it is a derivative of estradiol. Estradiol is converted to estriol in perchlorate exposed animals which leads to an increased estriol concentration and is responsible for the development of pseudopregnancy.

In PTU-induced or thyroidectomized female rats, the estrous cycle becomes irregular[28] In our present study, perchlorate in pharmacological doses has been administered daily for 28 days. The estrous cycle was found to be almost regular till 22 ± 2 days of perchlorate exposure, thereafter the reproductive cycles of the treated animals became irregular, followed by a consistent change of vaginal smear, showing an apparent stage of estrous as evident by the presence of cornified epithelial cells as found in this stage of estrous cycle in normal adult female rats. A similar finding was reported by Follett and Potts who found a prolonged estrous phase in hypothyroid ewes. In perchlorate exposed animals the ovary became hypertrophied as shown by an increase in its weight and histological architecture consistent with a proliferative uterus having numerous secretory glands and relatively high level of serum estriol.

Histological section of the perchlorate exposed ovary when compared with that of the control, while both in estrous phase reveals that in control rat, the ovary showed the presence of Graafian follicles with numerous antral and

pre-antral follicles whereas, the ovary of perchlorate exposed animals showed the appearance of many corpus luteum as found in pseudopregnancy. Therefore, perchlorate appears to develop a state of pseudopregnancy interfering at the level of the hypothalamic-pituitary-ovarian axis, which is under further investigation. The endometrial structure of the control animals showed a normal appearance, that is, the lumen in the centre, surrounded by numerous glands and thickened endometrium followed by myometrium; while the lumen of the perchlorate exposed animals showed additional enlargement of the uterus, followed by the presence of numerous secretory glands as found in pregnancy or pseudopregnancy.

The study elucidated that though perchlorate is used as an antithyroid agent in the treatment of hyperthyroidism/ thyrotoxicosis etc. but its action is quite different to that of any other antithyroid agents on ovarian morphology and functions. Perchlorate exposure gradually ceased the estrous cyclicity as well as hypertrophied and hyper-functional ovary and uterus as indicated by their structural features, and enhanced activity of steroidogenic enzymes resulting in the synthesis of more estriol as found in pregnancy presenting a steady estrous phase of vaginal smear. Therefore, prolonged exposure of perchlorate as used in this study gradually established enlarged ovary and uterus and their hyper-functional states as found in pseudopregnancy.

Conflict of interest

We do not have any conflict of interest for the present article.

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