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Reproductive toxicity of perchlorate in rats

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ABSTRACT

Perchlorate, as an oxidizer, has many applications such as explosives and pyrotechnics, especially in rocket propellants and missile motors. Because it was found in water including wells and drinking water in the US, its effect on human health was being noted. However, the reproductive toxic effect on perchlorate is still unclear. In present study, the effects of repeated exposure to perchlorate on reproductive toxicity were evaluated in Wistar rats. The rats were treated orally with perchlorate at doses of 0.05, 1.00 or 10.00 mg/kg body weight (b.w.) daily for 8 weeks. The levels of T_3 and T_4 hormones in the rat serum were detected by radioimmunoassay kit. The indexes of reproduction, percentage of organ in body weight (%) and frequency of abnormal sperm cells were also analyzed in this study. DNA damage in testicular cells was evaluated by Comet assay. The levels of MDA, GSH and SOD were examined in testicle tissues of rats by ELISA. The expression of c-fos and fas protein was examined in testicle tissues by immunohistochemistry. The results showed that perchlorate did not affect the body weight of rats. Perchlorate also significantly decreased indexes of live birth and weaning in the groups of 1.00 and 10.00 mg/kg, and viability index only in the 10.00 mg/kg group ($P < 0.05$). Perchlorate also significantly decreased the serum level of T_3 in male rats of 1.00 and 10.00 mg/kg groups, increased the rate of sperm abnormality (10.00 mg/kg), potentially caused DNA damage in testicular cells and altered the status of oxidative stress in male rats. In addition, because of the increase in the expression of fas and c-fos protein in testicle tissues, perchlorate could induce apoptosis in spermatogenesis. Thus, these findings indicate that perchlorate could cause DNA damage in testicular tissues and reduce testicular spermatogenic ability, resulting in reproductive toxicity.

1. Introduction

Perchlorate exists in the natural and man-made environment(Kim and Logan, 2001). As an oxidizer, perchlorate is used in explosives, pyrotechnics and lots of other fields, especially in rocket propellants and missile motors. Perchlorate also used to other applications such as Graves' disease and control hyperthyroid conditions (Bajuk et al., 2017; Okosieme and Lazarus, 2016). However, the sources of environmental contamination come from most of primary oxidants in fireworks (Wilkin et al., 2007). Because of highly soluble and stable in the atmosphere, perchlorate is immediately dissolved in the ground water. Thus, perchlorate is ubiquitous contamination in any places including soil and groundwater (Bernhardt et al., 2011; Ellington et al., 2001; Jackson et al., 2005; Liu et al., 2009; Yu et al., 2004). Perchlorate has recently noted in the public because of its discovery in the wells and drinking water in the North America and Asia (Backus et al., 2005; Kosaka et al., 2007; Shi et al., 2007; Srinivasan and Viraraghavan, 2009). Perchlorate is also found in many common products of household and industry (Dasgupta et al., 2005). Perchlorate also was widely found in food (Abt et al., 2018). This may increase the chance to expose human in daily life. Since perchlorate was detected in groundwater in 1950s, its effect on toxicology has only been found in the 2000s (Kirk, 2006; Lamm and Doemland, 1999). Because of its similarity of chemical structure with iodide, the main toxic effect of perchlorate on thyroid gland was noticed to affect iodide uptake (Burgi et al., 1974; Chen et al., 2014; Leung et al., 2010; Steinmaus et al., 2013; Trumbo, 2010).

Reactive oxygen species (ROS) is involved in DNA damage, resulting in the status of oxidative stress and possibly modulated the apoptotic pathway(Chandra et al., 2000; Gunes and Mahmutoglu, 2019; Milisav et al., 2017). However, oxidative stress is also related to the male reproduction(Gunes and Mahmutoglu, 2019). As a chemical and similar structure with iodide, perchlorate may first affect thyroid hormones (T_3

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Table 1
Body weight of rats fed with perchlorate during 8 weeks (mean ± S.D.).

Group (mg/kg b.w.)		the number of rats (n)	initial weight (g)	2^{nd} weeks (g)	4^{th} weeks (g)	6^{th} weeks (g)	8^{th} weeks (g)
10.00	Male	10	132.19 ± 27.51	193.87 ± 28.29	244.55 ± 42.59	281.45 ± 41.08	330.00 ± 47.81
	Female	10	125.38 ± 16.26	170.66 ± 15.48	208.73 ± 15.45	220.34 ± 18.16	237.82 ± 21.28
1.00	Male	10	126.26 ± 21.45	197.24 ± 24.78	260.55 ± 41.56	293.88 ± 44.32	332.25 ± 40.68
	Female	10	120.27 ± 6.54	165.36 ± 15.77	194.25 ± 16.16	217.46 ± 16.44	235.22 ± 19.64
0.05	Male	10	131.25 ± 20.02	190.66 ± 29.86	247.56 ± 35.65	280.14 ± 40.34	310.55 ± 40.20
	Female	10	123.41 ± 20.35	163.85 ± 17.06	203.34 ± 14.36	218.03 ± 11.55	238.18 ± 13.42
Control	Male	10	125.44 ± 22.12	188.86 ± 30.25	257.29 ± 32.48	290.28 ± 34.58	330.05 ± 36.05
	Female	10	130.53 ± 19.86	175.82 ± 19.02	204.14 ± 23.55	215.48 ± 27.44	234.80 ± 21.23

Table 2
The indexes of mainly organs by body weight in rats treated with perchlorate (mean ± S.D.).

Group (mg/kg b.w.)		the number of rats (n)	liver index (%)	spleen index (%)	kidney index (%)	thyroid index (%)	testis index (%)	ovary index (%)
10.00	Male	10	4.24 ± 0.42	0.30 ± 0.05	0.70 ± 0.04	0.19 ± 0.05	0.92 ± 0.10	
	Female	10	4.26 ± 0.22	0.38 ± 0.14	0.71 ± 0.05	0.22 ± 0.03		0.06 ± 0.02
1.00	Male	10	4.46 ± 0.25	0.26 ± 0.15	0.75 ± 0.08	0.24 ± 0.05	0.90 ± 0.11	
	Female	10	4.28 ± 0.45	0.34 ± 0.05	0.84 ± 0.14[a]	0.23 ± 0.06		0.06 ± 0.01
0.05	Male	10	4.28 ± 0.65	0.27 ± 0.10	0.78 ± 0.10	0.21 ± 0.05	0.95 ± 0.21	
	Female	10	4.40 ± 0.36	0.32 ± 0.06	0.74 ± 0.08	0.23 ± 0.05		0.06 ± 0.01
Control	Male	10	4.50 ± 0.68	0.25 ± 0.07	0.77 ± 0.12	0.21 ± 0.04	0.98 ± 0.20	
	Female	10	4.48 ± 0.57	0.35 ± 0.18	0.72 ± 0.04	0.22 ± 0.05		0.06 ± 0.02

[a] $P < 0.05$, when compared to the control group.

Table 3
The indexes of reproduction in rats treated with perchlorate (mean ± S.D.).

Group (mg/kg b.w.)	the number of rats (n)	mating index (%)	fertility index (%)	gestational index (%)	live birth index (%)	viability index (%)	weaning index (%)
10.00	10	80	90	80	70.80 ± 7.74*	39.30 ± 5.45*	20.80 ± 6.30*
1.00	10	70	60	70	71.90 ± 8.60*	47.50 ± 6.41	43.80 ± 8.24*
0.05	10	80	80	70	73.30 ± 7.44	50.80 ± 7.02	49.17 ± 8.45
Control	10	100	80	90	87.80 ± 6.56	69.40 ± 10.67	64.30 ± 8.30

*$P < 0.05$, **$P < 0.01$, when compared to the control group.

and T_4). The triiodothyronine (T_3) and thyroxine (T_4) are secreted from the thyroid gland. The feedback mechanism controls the synthesis and secretion thyroid hormones (Larsen, 1982). Once serum levels of T_3 and T_4 are decreased, thyrotropin-releasing hormone (TRH) and thyroid stimulating hormone (TSH) are secreted and released by the hypothalamus and anterior pituitary, which promote iodide uptake and synthesis of thyroid hormones (Larsen, 1982). The levels of T_3 and T_4 rising at a certain degree can reduce in the levels of TRH and TSH, which are the negative feedback(Larsen, 1982). Overloaded perchlorate in the environment leads to a competitive inhibition of iodine taken to affect the function of thyroid(Larsen, 1982). The inhibition of iodide taken is considered as main effects of perchlorate, which decrease levels of T_3 and T_4 in the serum, and increase the TSH level resulting in hypothyroidism(Zhong et al., 2018), especially affecting the fetal neurodevelopment(Alexander et al., 2004; Cao et al., 1994; Haddow et al., 1999). Available data show that thyroid hormones are not only involved in the testis development, but they also help to maintain the testicular physiology(Sahoo et al., 2019). However, the effects of perchlorate on reproductive toxicity are still unclear. Based on this limited information, the aims of this study were to determine the reproductive toxicity of perchlorate through the frequency of abnormal sperms, Comet experiment, oxidative damage, the expression of Fas and c-fos protein in Wistar rats.

2. Materials and methods

2.1. Dosage selection

The levels of perchlorate dosage in this study were selected according to the literature (Ferlay et al., 2019; Greer et al., 2002; Gurunathan et al., 2018). Due to oral administration of ammonium perchlorate (10 mg/kg/day) altering the weight of thyroid organ in a rat study and the recommendation of perchlorate RfD (0.0007 mg/kg/day) by EPA, the dosage selection of this study was to further identify the effect of perchlorate at doses of 0.05, 1.00 and 10.00 mg/kg body weight (b.w.) on the reproductive system.

2.2. Animal experiments

Wistar rats (n = 80, 6 weeks old, weighing about 80–100g) were purchased from Experimental Animal Center of Basic Medical School of Jilin University, and housed in a room maintaining at 22 ± 2 °C with a 12h: 12h (light: dark) cycle. Rats were permitted to acclimate the surroundings for 1 week. Adult male (n = 40) and female (n = 40) rats were kept in large cage separately. The rats were randomly divided into the control or three treatment groups. Ten male and 10 female rats were allocated in each group. All the rats were daily given water, 0.05, 1.00 or 10.00 mg/kg b.w. of sodium perchlorate solution (100% purity, Guangfu Chemical Co. Ltd.) by gavage for 8 weeks. The rats in estrus were then allowed to mate for 1 week (one male and one female per cage). Male rats were given the sodium perchlorate solution until mating over, and female rats were also given the sodium perchlorate solution until weaning of the offspring. The body weight of rats was weighted weekly. The indexes of evaluation in reproduction such as the mating index (the ratio of the number of females was checked out the sperms in the vagina after mating), gestational index (the ratio of the number of pregnant females after mating), fertility index (the ratio of the number of normal puerperal females with pregnant females), live birth index (the ratio of the number of born alive offspring with the total number born), viability index (the ratio of the survival number of

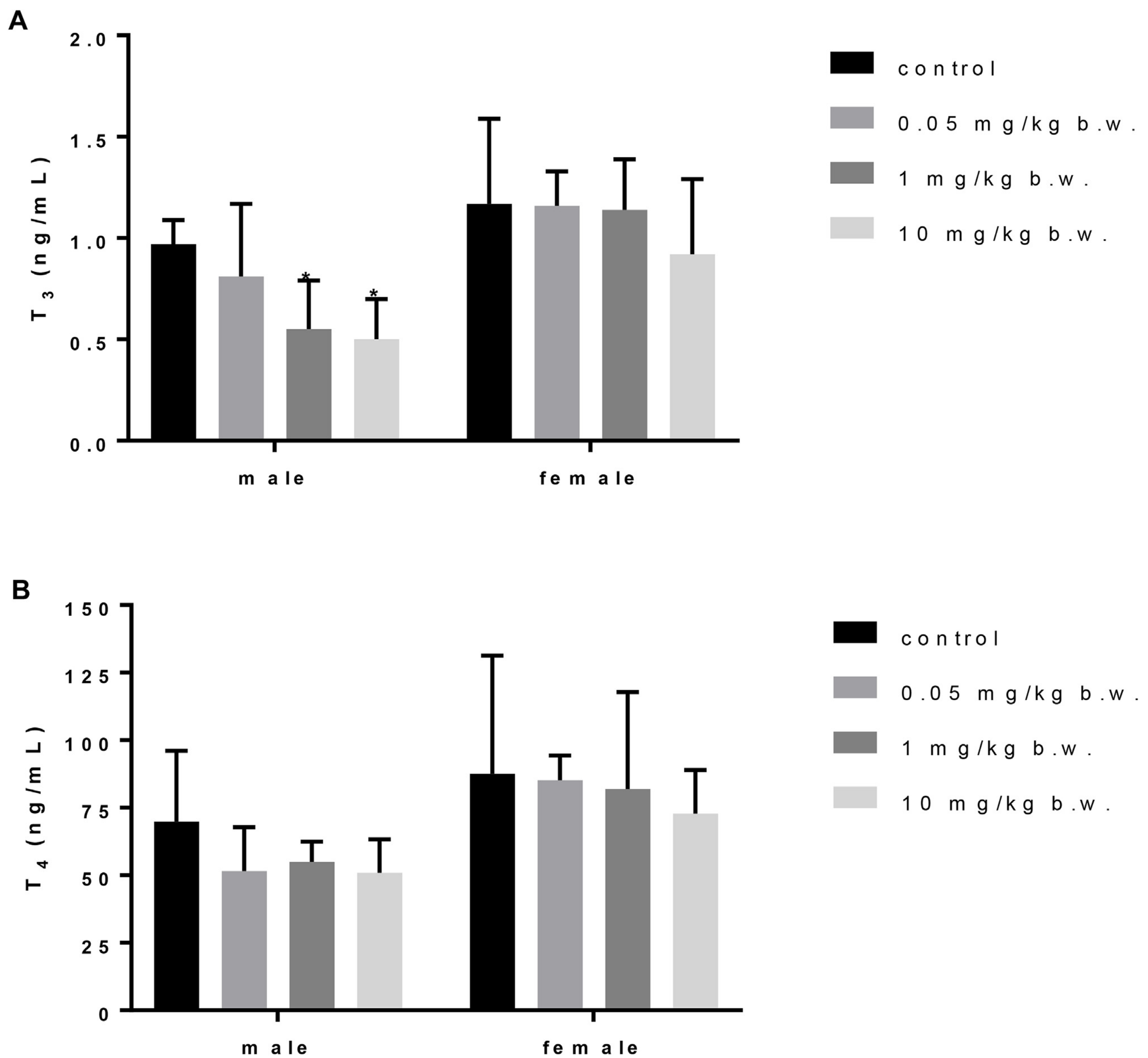

Fig. 1. The serum levels of T_3 and T_4 in rats treated with perchlorate. The data are expressed as mean ± standard deviation (S.D.) (n = 10). *$P < 0.05$, when compared to the control group.

offspring after 4 days lactation with and the number of born alive offspring) and weaning index (the ratio of the weaning number of offspring with the total number of born alive offspring) were calculated in the whole experiments. All protocols for animal care and experiments were approved by the Ethics Committee of Harbin Medical University (Harbin, China).

2.3. Determination of rat organ coefficient

After rats were fasted except for water for 12 h, the rats were sacrificed and blood was collected from the abdominal aorta under CO_2 at the termination of the experiment. The wet weight of organs was weighed at the time of necropsy and then calculated the organ coefficients (organ weight/body weight × 100).

2.4. Hormone assay

The collected blood was placed for 10 min at room temperature and then centrifuged at 2500 rpm for 15 min. Serum levels of T_3 and T_4 were measured using a radioimmunoassay kit (Beijing North Biotechnology Institute, Beijing).

2.5. Sperm abnormality test

After perchlorate administration, the epididymis in each male rat was carefully removed and cut in a plate with saline, then filtered with a clean len-paper. The sperm slides were made from the filtrate at least triplicate in each male rat. The slides were dried in the air, fixed with methanol for 5 min and then stained with 1% eosin for another 15 min. The 1000 complete sperms for each male rat were observed at random under the microscope (× 400) and then counted the number of abnormal sperms.

2.6. Comet assay

2.6.1. Preparation of testicular cell suspension

Testicles were put into a plate and washed twice with pre-warmed D-Hanks solution (37 °C). The capsules and blood vessels in testicles

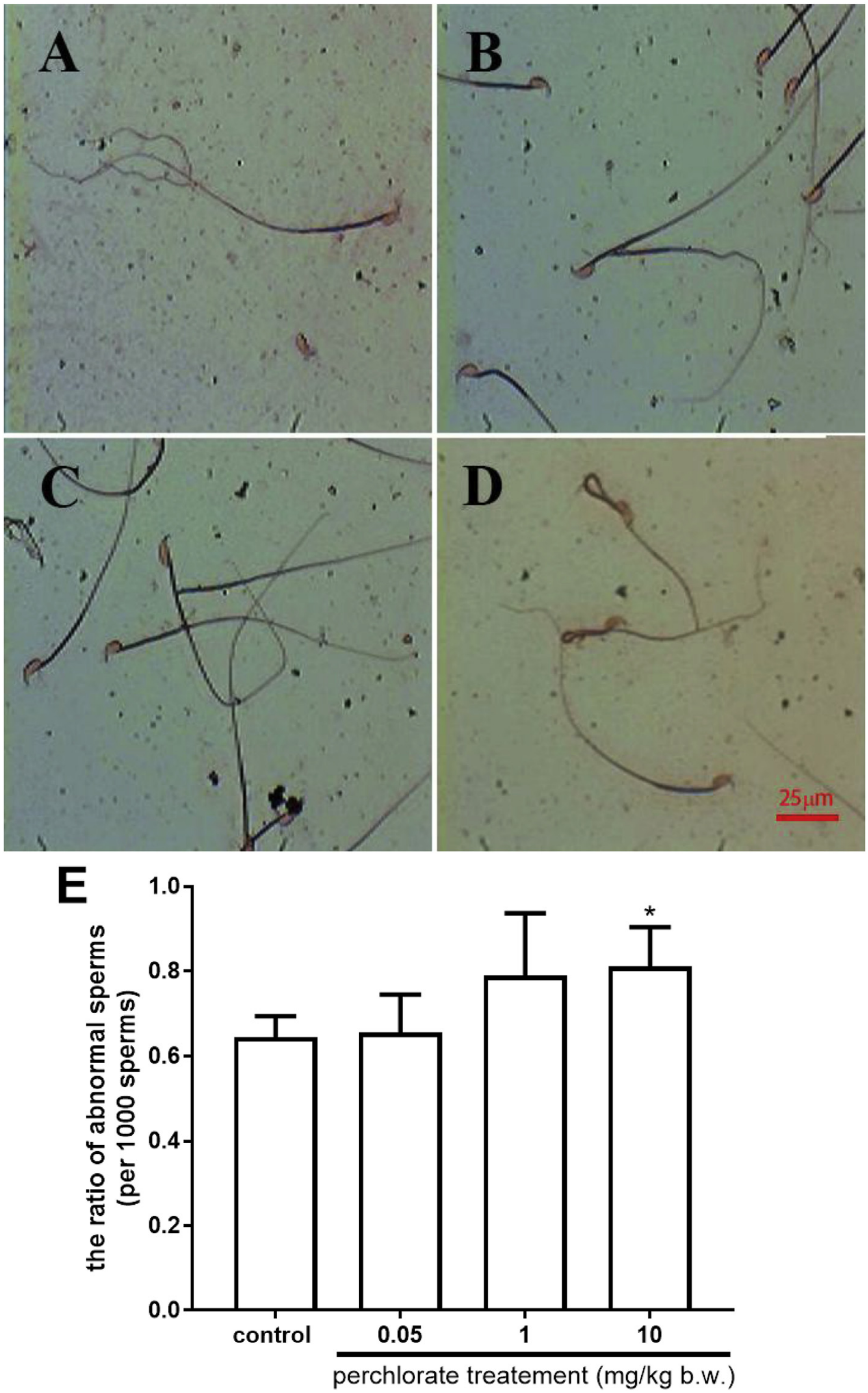

Fig. 2. Abnormal ratio of sperms in rats treated with perchlorate. **A** and **B** show double tails of germ; **C** and **D** show folding tails of germ. **E** shows the frequency of abnormal sperms (per 1000 sperms) in 7 rats. $^{*}P < 0.05$, when compared to the control group.

were removed carefully and then transferred into a small beaker which was filled with 2 mL D-Hanks solution. Seminiferous tubules were cut into small pieces with a ophthalmic scissor as soon as possible. The solution containing pieces was diluted by D-Hanks solution. The mixed solution was filtered through a 300 mesh stainless steel filter. The cell suspension was collected. The live cells were counted by trypan blue staining. When live cells were over 90% in the cell suspension, the cell suspension was kept at 4 °C to use for the silver staining assay.

2.6.2. Comet assay - silver staining

The comet assay was performed in our previous study (Yu et al., 2013). The stained slides were coded blindly at random and reviewed under a microscope. One hundred cells from coded slides were analyzed in each rat. The number of cell with comet tail DNA was counted and extent tail moment of cells was measured from seven rats.

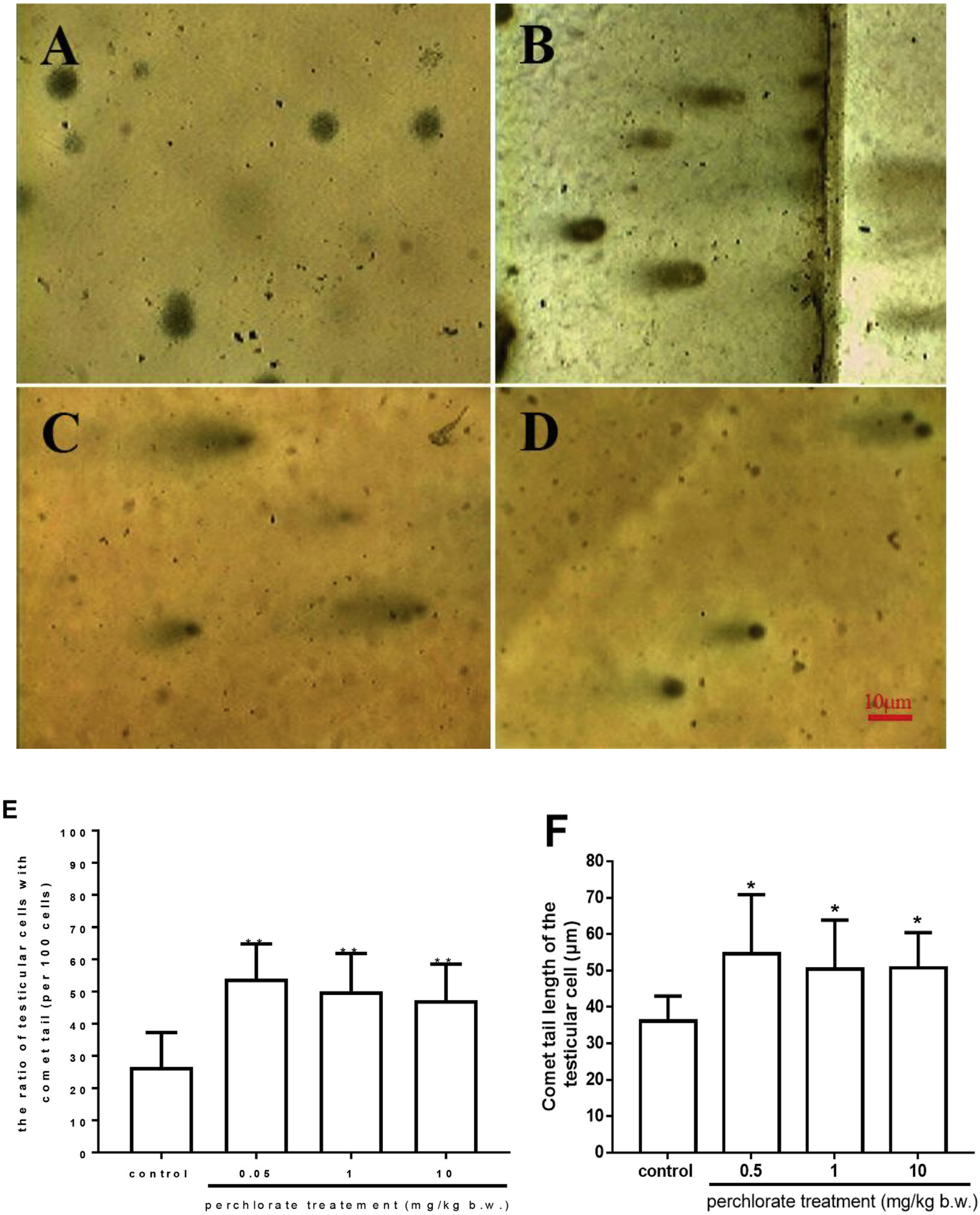

Fig. 3. The ratio of testicular cells with comet tail and length of comet tail in male rats treated with perchlorate. **A** Cells in the control group; **B** Cells in the 0.05 mg/kg b.w. group; **C** Cells in the 1.00 mg/kg b.w. group; **D** Cells in the 10.00 mg/kg b.w. group; **E** and **F** show the ratio of testicular cells with comet tail and length of comet tail in 7 rats (mean ± S.D.). $^{*}P < 0.05$, $^{**}P < 0.01$, when compared to the control group.

2.7. Biochemical analysis

Testicles were put into a plate and washed with cold saline twice, and then carefully removed the capsules and blood vessels as described before. Testicles were put into 1.8 mL cold saline, cut into pieces using an ophthalmic scissor and ground with a homogenizer for 6–10 times. After centrifuged at 2500 rpm for 20 min, the supernatant was collected and then diluted 10 times using saline for malondialehyde (MDA) and 100 times for superoxide dismutase (SOD) as well as total protein detection. The level of glutathione (GSH) was determined in the supernatant. The levels of MDA, SOD and GSH were measured as described as before (Liu et al., 2010). The content of MDA, the activity of SOD and GSH were expressed as nmoles or units (U) per mg total protein (prot).

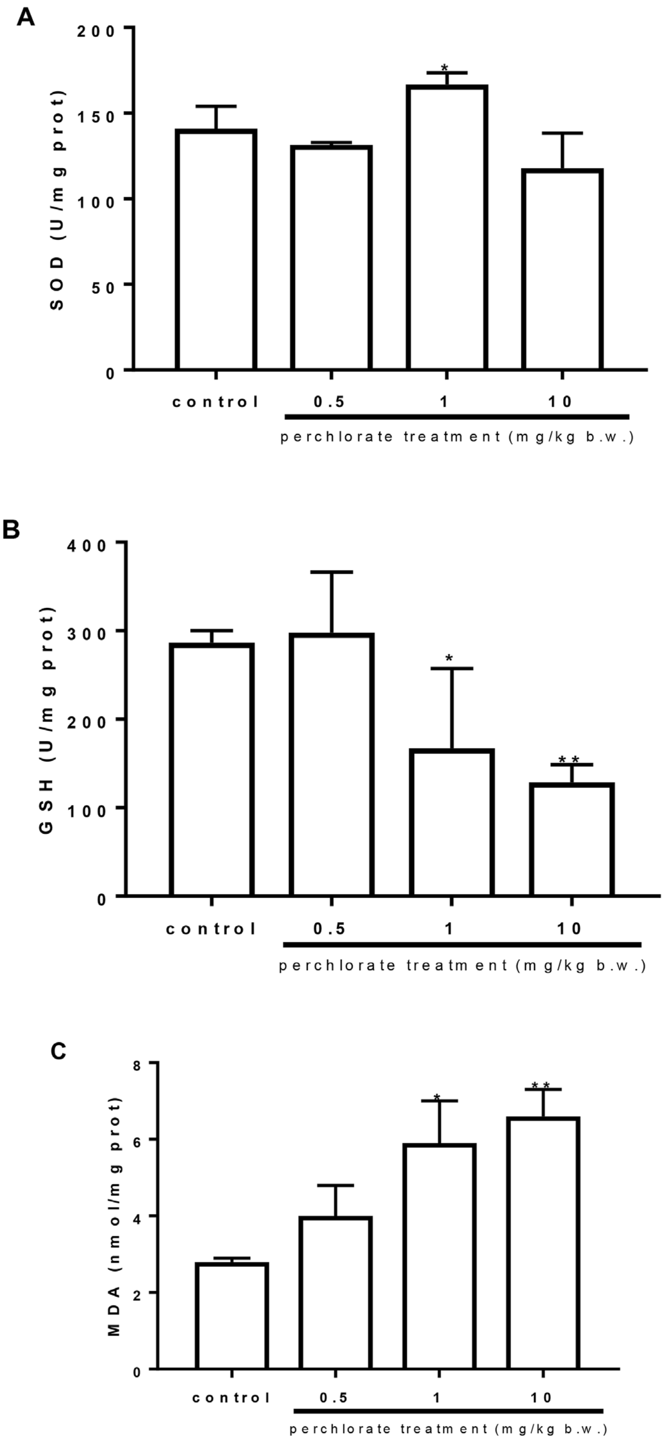

Fig. 4. The levels of SOD, GSH and MDA in testicle tissues of rats treated with perchlorate. The data are expressed as mean ± S.D. (n = 7). *$P < 0.05$, **$P < 0.01$, when compared to the control group.

2.8. The expression of fas and c-fos protein in testicular tissues

After sacrificed the rats under CO_2, one of testicles was fixed into 10% formalin for 48 h, graded dehydrate using alcohol, embedded into wax and final made paraffin sections. The expression of fas and c-fos protein was determined in testicular tissues by immunohistochemistry as described studies(Liu et al., 2008; Wang et al., 2013). Briefly, after hydrated by graded alcohol to distill water, the slides were put into the 3% H_2O_2 for 10 min in order to inactivate endogenous enzyme, and then washed twice in distilled water. After added 10 mmol/L citrate buffer (pH 6.0), the slides were heated to boiling in the microwave and then cooled down at the room temperature. After washed in PBS 3 times (5 min/time), non-specific goat serum was added into the slides to block non-specific binding. The slides were incubated with anti-c-fos or anti-fas primary antibodies overnight at 4 °C. After washed 3 times using PBS, the slides were incubated secondary HRP labeled anti-mouse antibody (ZYMED Lab, Carlsbad, CA) for 30 min. After washed with PBS for 3 times, the slides were added 3, 30-diaminobenzidine tetrahydrochloride (DAB) to develop the chromogenic reaction under a microscope. All slides were counterstained with hematoxylin for 20 s. The negative controls also were performed to the same protocol without the primary antibody. Images were taken under microscope (× 400). The positive cells were counted in the slides randomly selected from 5 rats in each group. The results were expressed as the number of positive cells per field.

2.9. Statistical analysis

The data are expressed as mean ± standard deviation (S.D.). The differences were analyzed using a SPSS for Windows 23.0 (IBM, Armonk, NY). After tested for the normal distribution by the Shapiro-Wilk test, the data were analyzed by Kruskal-Wallis test or One-way ANOVA and followed by Duncan's test for group's comparison. *P* value was less 0.05 as a statistical significance.

3. Results

3.1. Indexes of reproduction

The changes of body weight in different weeks are shown in Table 1. There were no differences both in male and female between perchlorate treatment and control groups ($P > 0.05$). The mainly organs were weighted and organ indexes i.e. the coefficients were calculated in rats treated with perchlorate. As shown in Table 2, the coefficient of kidney in female rats of 1.00 mg/kg perchlorate group was significantly different from the control group ($P < 0.05$). The coefficients of liver and spleen in the perchlorate treatment groups were not different from the control group ($P > 0.05$). The coefficient of thyroid in the perchlorate treatment groups was also not different from the control group ($P < 0.05$). However, although there was no difference between perchlorate treatment and control groups, the testis coefficient in the perchlorate treatment group was a little low when compared to the control group. No difference was found the coefficients of ovary between the perchlorate treatment and control groups ($P > 0.05$).

To further determine whether perchlorate affects the function of reproduction in rats, the reproductive parameters were calculated. As shown in Table 3, indexes of mating, fertility and gestation in rats fed with perchlorate were not different from the rats in the control group ($P > 0.05$). However, indexes of live birth, viability and weaning were significantly different in perchlorate treatment groups ($P < 0.05$).

3.2. The serum levels of T_3 and T_4 in rats

To further determine whether perchlorate also affects the reproductive function because of decreased indexes of live birth, viability and weaning, serum levels of T_3 and T_4 reflecting thyroid function, were first examined in male and female rats. As shown in Fig. 1, the level of T_3 in male rats was significantly decreased in the perchlorate treatment groups when compared to the control group in a dose-dependent manner ($P < 0.05$). No difference was found in the levels of T_3 and T_4 in female rats between the perchlorate treatment and control group ($P > 0.05$).

3.3. The morphological changes of sperm

From the results of T_3 and T_4 levels, perchlorate could affect male

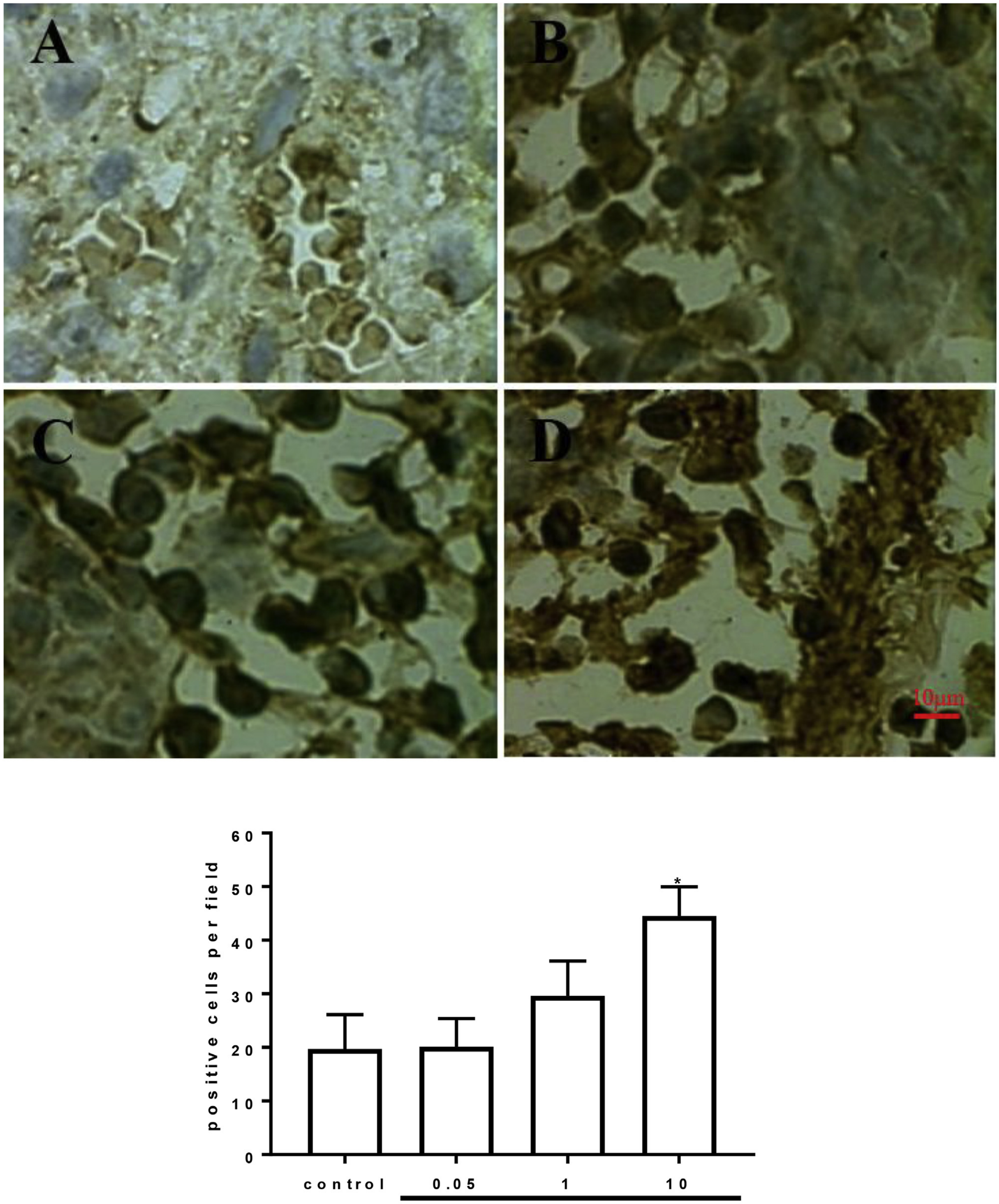

Fig. 5. The expression of Fas protein in testicular tissue in rats treated with perchlorate. **A** shows the expression of Fas protein in the control group; **B** shows the expression of Fas protein in the 0.05 mg/kg b.w. group; **C** shows the expression of Fas protein in the 1.00 mg/kg b.w. group; and **D** shows the expression of Fas protein in the 10.00 mg/kg b.w. group. The results were expressed as mean ± S.D.(n = 5). The expression of fas protein in germ cells was significantly increased in perchlorate treatment groups in comparison with the control group (*$P < 0.05$).

thyroid function. To further determine whether perchlorate also affects the reproductive function of male rats, the morphological changes of sperm were examined. As shown in Fig. 2, the morphological changes of sperm in male rats treated with perchlorate often observed double tails and folding tail of sperm, a few sperms in double heads. The ratio of abnormal sperms in male rats treated with perchlorate were

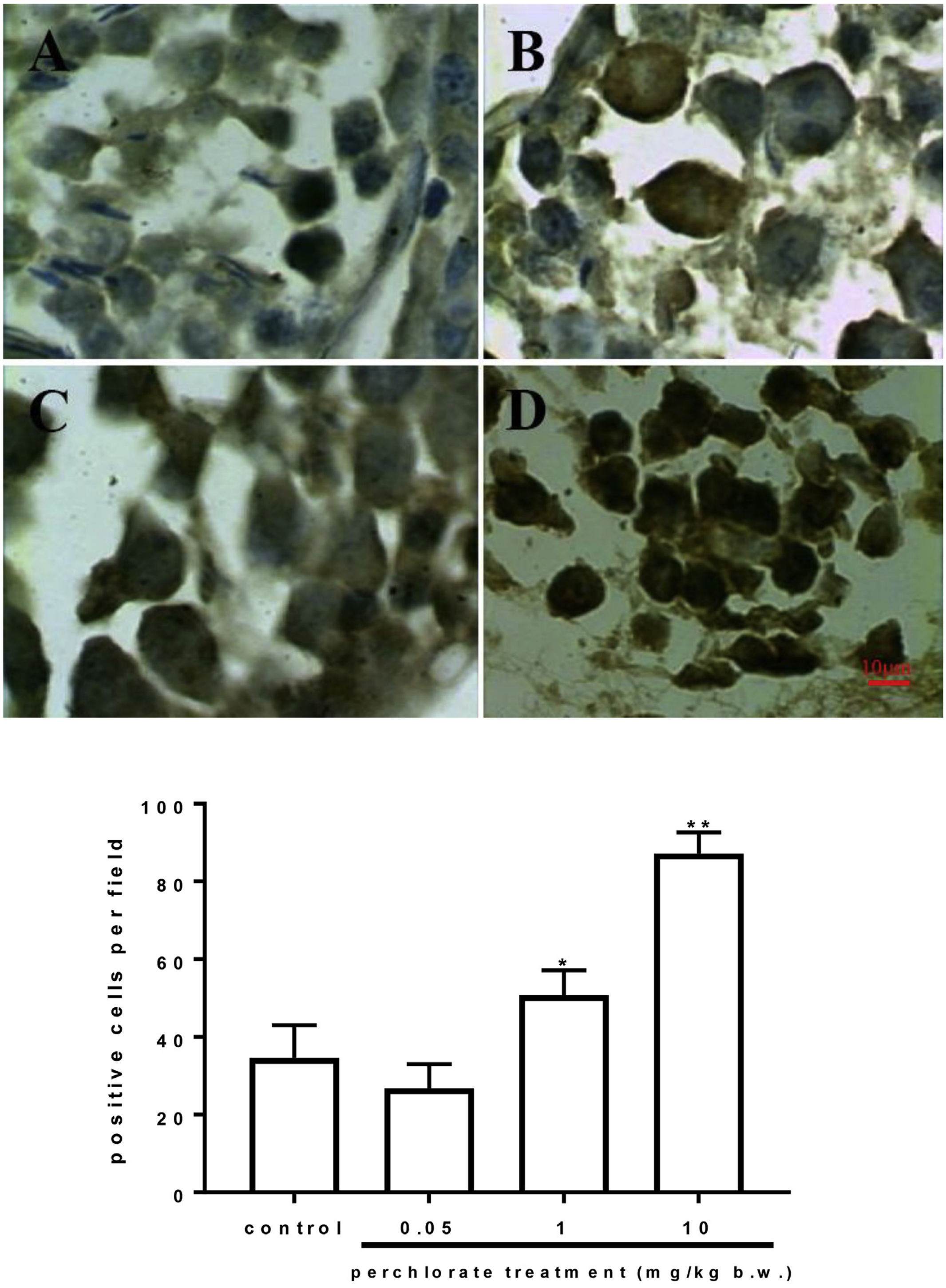

Fig. 6. The expression of c-fos protein in testicular tissue in rats treated with perchlorate. **A** shows the expression of c-fos protein in the control group; **B** shows the expression of c-fos protein in the 0.05 mg/kg b.w. group; **C** shows the expression of c-fos protein in the 1.00 mg/kg b.w. group; and **D** shows the expression of c-fos protein in the 10.00 mg/kg b.w. group. The results were expressed as mean ± S.D.(n = 5). The expression of c-fos protein in germ cells in perchlorate treatment groups was significantly increased when compared to the control group (*$P < 0.05$ and **$P < 0.01$).

significantly higher than that in the control group in a dose-dependent manner ($P < 0.05$) (Fig. 2).

3.4. DNA damage and oxidative stress of testicular cells

The DNA damage of testicular cells was determined by Comet assay via the comet tail rate and tail length of cells. As shown in Fig. 3, the rates of cells with comet tail and length of comet tail in testicular cells in perchlorate treatment groups were significantly increased in comparison with the control group ($P < 0.05$ and $P < 0.01$). The maximum of comet tail rate and comet tail length of testicular cells were found at dose of 0.05 mg/kg b.w. perchlorate group (Fig. 3). The oxidative stress of testicle also was measured in the rat serum. The level of malondialehyde (MDA) in testicles of rat, reflecting the status of lipid peroxidation in cells, was significantly increased in the perchlorate treatment groups in a dose-dependent manner ($P < 0.05$) (Fig. 4C).

Oppositely, the system of anti-oxidation, such as superoxide dismutase (SOD) and glutathione (GSH), was also measured in testicles of rat. The level of GSH was significantly decreased in perchlorate treatment groups in a dose-dependent manner ($P < 0.05$ and $P < 0.01$) (Fig. 4B). No difference was found the level of SOD in perchlorate treatment groups except for the 1 mg/kg perchlorate group when compared to the control group (Fig. 4A).

3.5. The expression of fas and c-fos protein in testicular tissues

In order to further determine whether apoptotic signaling pathways are also involved in DNA damage in testicular cells, the expression of fas and c-fos protein was examined in testicular tissues by immunohistochemistry. As shown in Fig. 5, the expression of fas protein in germ cells was significantly increased in perchlorate treatment groups in comparison with the control group ($P < 0.05$). The expression of c-fos protein in germ cells in perchlorate treatment groups was also significantly increased when compared to the control group ($P < 0.05$ and $P < 0.01$) (Fig. 6). Both total positive number of fas and c-fos in germ cells showed a dose-response.

4. Discussion

Perchlorate was a widespread endocrine disruptor that was previously correlated with increasing serum level of TSH and decreasing levels of thyroid hormones both in animals and human (Gurunathan et al., 2018). Oral administration of ammonium perchlorate to rats could increase the weight of thyroid organ, which corresponding to histopathological changes in the thyroid after 14 and 90 days at a dosage level of 10.0 mg/kg/day. The difference was observed the changes of serum TSH and other thyroid hormones at a dosage as low as 0.01 mg/kg/day; however, no effects on thyroid organ weight or histopathological changes were observed at a dosage of 1.0 mg/kg/day (Ferlay et al., 2019). EPA has established perchlorate at an oral reference dose (RfD) (0.0007 mg/kg body weight per day) and a drinking water equivalent level (DWEL) (24.5 μg/L) according a previous study (Greer et al., 2002), in which healthy men and women were administered perchlorate from 0.007 to 0.5 mg/kg/day for a 14 day study. Thus, doses of 0.05, 1.0 and 10.0 mg/kg b.w. perchlorate were used in this animal study. In this study, perchlorate can affect the levels of thyroid hormones, which plays a critical role in maintaining the growth and development of body. Inadequate or excessive secretions of thyroid hormones also affect the body's growth and development(York et al., 2001). However, thyroid hormones (TH) direct affect the development of reproduction appearing an important regulator of testis development and testicular physiology(Sahoo et al., 2019). In this study, rats were given perchlorate at 0.05, 1.0 and 10.0 mg/kg/b.w. daily for 8 weeks, the indexes of live birth, viability and weaning were significantly decreased in perchlorate treatment groups in comparison with the negative control group ($P < 0.05$). These findings indicate that perchlorate could be through the placenta and lactation to affect the survival rate of young rats. It suggests that perchlorate might affect the reproductive function. Furthermore, the levels of TH in the rat serum were also determined in this study. The level of T_3 was significantly decreased in male rats of perchlorate treatment groups. It might feed perchlorate to disturb the iodine uptake to thyroid gland, resulting in a decreasing serum level of T_3 in male rats. In previous studies(Bajuk et al., 2017; Okosieme and Lazarus, 2016), perchlorate could cause iodide uptake inhibition, which decreases serum levels of T_3 and T_4. In another study (York et al., 2001), SD rats were given ammonium perchlorate in their drinking water at doses of 0, 0.3, 3.0, and 30.0 mg/kg/day in a two-generation reproduction study. The serum level of T3 was significantly increased in male rats at the dose of 30 mg/kg. These findings suggest that perchlorate administered to the rats in the drinking water at doses up to 30.0 mg/kg/day was not a reproductive toxicant, but it could affect the thyroid function at doses over 3.0 mg/kg/day. These data further indicate that perchlorate may interfere iodide uptake to thyroid gland. The productive function of male rats was further determined in this study. The morphological changes of sperms were examined in male rats. The ratio of abnormal sperms was significantly increased in male rats of perchlorate treatment groups. This can explain the reason why the indexes of live birth, viability and weaning were decreased in rats fed with perchlorate, indicating that perchlorate had the reproductive toxicity.

To further explore effect of perchlorate on the reproductive toxicity and its possible mechanism, DNA damage was examined in germ cells by single cell gel electrophoresis assay. Single cell gel electrophoresis (SCGE) technique, as called comet assay, is an assay for detecting DNA damage and repair of eukaryotic cells in a single cell level. In present study, comet tail rate and length of comet tail in the testicular cells were significantly increased in perchlorate treatment groups. It indicated that perchlorate could cause DNA damage in testicular cells. In previous studies(Siglin et al., 2000; Yu et al., 2007), the genotoxicity of perchlorate has been evaluated by in vitro and in vivo assays. Sodium perchlorate not only caused the negative results of mutagenicity in the Ames assay (with or without S9 activation) starting from 1.0 mg to 5.0 mg(Yu et al., 2007), but it also induced the negative results of chromosomal anomalies in the bone marrow of mice starting at 283.0 mg/kg, 566.0 mg/kg for female mice and 394.0 mg/kg, 788.0 mg/kg for male mice(Yu et al., 2007). Ammonium perchlorate in the drinking water at dosage levels of 0.01, 0.05, 0.2, 1.0, and 10.0 mg/kg/day was evaluated the subchronic toxicity when administered to SD rats for 14 or 90 days (Siglin et al., 2000). No increase rate of micronuclei was found at the dose of 10.0 mg/kg/day, which produced both thyroid hormone perturbations and follicular cell hyperplasia.

Although perchlorate could induce DNA damage in testicular cells, we further determine whether reactive oxygen species (ROS) was also involved in this damage. DNA damage is caused by some harmful factors produced in the metabolic process of the body. Endogenous injury factors mainly included free radicals i.e. ROS and advanced glycation end-products (AGE), etc. Under physiological conditions, the body has a set of complete antioxidant mechanism of elimination of free radicals, which can be rapidly cleaned and quenched by the antioxidant enzymes and antioxidants avoiding damage DNA of cells. However, when the body produces too much free radicals or declines antioxidant function, free radicals will have damage to DNA. Under normal conditions, oxidative damage and antioxidant defense system were in a state of dynamic balance in the cells. When affected by environmental toxins, due to overload of oxidation and the depletion of antioxidants, balance is destroyed, resulting in oxidative stress to induce lipid peroxidation and/or DNA damage. Many studies have been shown that the clean systems of ROS include superoxide dismutase (SOD), glutathione peroxidase glycosides peptide, catalase and the peroxidase family in the body. These changes in the enzymatic content and activity directly affect the content of ROS in the cell. The vitality of SOD indirectly reflects the ability to scavenge oxygen free radicals. Lipid peroxide is a product that oxygen free radicals attack unsaturated fatty acids in the biofilm formed, which can produce the polymer. Polymers react with the protein and DNA in the human body, resulting in the variation of the protein structure and loss of normal function of the mutant protein, which can lead to cancer ultimately. Detection of malondialehyde (MDA) can reflect the level of lipid peroxidation and the degree of cell damage affected by free radicals in the body. GSH-Px is a kind of important catalytic decomposition of hydrogen peroxide enzyme widespread within the body. The reduction reaction of hydrogen peroxide reduced glutathione (GSH), which can protect the cell membrane structure and function. In our study, perchlorate significantly increased the concentrations of MDA and significantly decreased the GSH level in testicle tissues. These data suggested that perchlorate could cause ROS in testicle tissues, resulting in DNA damage.

To further identify whether apoptotic signal pathways were participated in the sperm abnormality, the expression of fas and c-fos protein was determined in germ cells. Fas is a kind of cell surface proteins, expressed in the thymus cells and activated in lymphocytes, partly in tumor cells or other cells or tissues. Fas can combine with Fas-L and induce cell apoptosis, and it has a very close relationship with a variety of functions and the diseases in the body (Francavilla et al., 2002; Richburg et al., 1999). A normal process of spermatogenesis exist obvious apoptosis in mammalian (Ferlay et al., 2019). When the mature period of spermatogenic cells was blocked, the apoptosis rate of spermatocyte was raised and the expression of Fas and Fas-L was increased in spermatocyte(Bhardwaj and Aggarwal, 2003; Carey et al., 2000; Eguchi et al., 2002; Philchenkov, 2003). C-fos proto-oncogene gene belongs to the Immediately Early Gene (IEG) family. C-fos can make quick response to external stimuli, and it is considered as the important role “third messenger” within the nucleus in the process of information transfer (Bullitt, 1990; Giovannelli and Bloom, 1992; Nic Dhonnchadha et al., 2012). C-fos proto-oncogene gene expression has tissular, cellular and staged specificity. In the early embryonic development, c-fos is only expressed in amniotic membrane and placenta, while in postembryonic period, expressed in the developmental central nervous system, embryonic bone, and cartilage and teeth growing region(Chen et al., 2016; Lacouture and Sibaud, 2018; Nallasamy et al., 2018). In adult organs, c-fos is expressed in some specific hematopoietic cells in bone marrow cells, such as macrophages and granulocyte, also expressed in reproductive cells. But in most other cells, its expression level is relatively lower, and it needs to be stimulated to maintain the high expression of c-fos(Barr, 2011). In recent years, the expression of c-fos proto-oncogene gene is increased in the process of the secretion of testosterone in leydig cells promoted by gonadotropins (Patel et al., 2017). A study speculated that the c-fos gene may regulate gene expression during the period of transition of germ cell development process according the high expression of c-fos in mouse spermatogonium. A large number of growth factors and hormones were produced in spermatogenesis process, and these bioactive substances can induce the c-fos gene expression in germ cells, which confirmed that the c-fos gene was closely associated with spermatogenesis function(Lacouture, 2006). In present study, the expression of fas and c-fos protein in germs cells was significantly increased in perchlorate treatment groups in a dose-response. These findings suggested the positive relationship between the high expression of fas and c-fos protein and testicular tissues induced by perchlorate. Thus, the effect of perchlorate on reproductive toxicity induce the changes of fas and c-fos protein.

5. Conclusions

In summary, our findings showed that although perchlorate did not affect the body weight, it could affect the indexes of live birth, viability and weaning, suggesting that perchlorate could reduce the reproduction. Our findings also found that perchlorate could decrease the serum level of T3 in male rats, indicating that perchlorate might affect the reproductive function of male. Further collected data showed that perchlorate caused the oxidative stress in testicular tissues by increased the MDA level and decreased levels of SOD and GSH, lead to DNA damage in testicular cells and induced apoptosis in sperm cells via increased the expression of fas and c-fos protein.

Conflict of interest statement

All authors declare that they have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.04.014.

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