Neuroendocrinology Letters ISSN 0172–780X


ORIGINAL ARTICLE

# Hypoxia alters testis development in neonatal rats

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*Submitted:* *April 1, 2002*
*Accepted:* *April 9, 2002*

*Key words:* **hypoxia; testis; postnatal development; testosterone; mitochondria**

*Neuroendocrinology Letters 2002; **23**:231–237 pii: NEL230302A05* 

**Abstract**

**OBJECTIVE:** To investigate the effects of continued hypoxia on postnatal development of the rat testis.

**DESIGN**: Wistar rats were exposed to simulated hypoxia from birth to postnatal day (PND) 45. Testosterone (T) in the plasma and the testis was measured in rats at PND 3, 7, 14, 21, 28, 35 and 45 respectively. Testis histology and cellular ultrastructure were studied.

**RESULTS**: a) Hypoxia induced a significant arrest of testes weight gain after PND 28; b) T release was significantly attenuated after PND 21; c) Alterations in histology and cellular ultrastructure were found in the testis, showing the swelling of testis interstitium and the enlargement of mitochondria in Leydig cells.

**CONCLUSION**: Postnatal hypoxia stress alters testis development both in terms of function and structure, especially at the critical age of gonadal development.

ABBREVIATIONS

| | |
|---|---|
| GH | Growth hormone |
| HPA axis | Hypothalamic-pituitary-adrenal axis |
| PND | Postnatal day |
| RIA | Radioimmunoassay |
| SHRP | Stress hyporesponsive period |
| T | Testosterone |

## Introduction

At high altitude, hypoxia is the critical stress factor influencing reproductive health. During the past decades, a number of studies have dealt with growth, development and reproduction under hypoxic conditions [1–8]. High altitude hypoxia delays onset of puberty in female rats [4–6]. In adult male rodents, hypoxia inhibits gonadotropins synthesis and release [9,10]. Chronic hypoxia induces a decrease in plasma testosterone (T) level [11], without changing T biosynthesis by the testis *in vitro* [12]. Moreover, chronic hypoxia arrests spermatogenesis in rats [13] and monkeys [14]. However, studies on the effects of hypoxia on testis development are few. With the increasing human activities at the Qinghai-Tibet Plateau in China, there is a growing concern about the reproductive development and performance at high altitude. The current study was designed to investigate the effects of hypoxia on development of the rat testis from the neonatal to reproductively mature ages.

## Material and methods

*Animals*: *Wistar* rats were provided by the Experimental Animals Centre, Northwest Plateau Institute of Biology, the Chinese Academy of Sciences (Xining). The strain had breed at the local altitude of 2.3 km (16% $O_2$, 77.33 kPa, $pO_2$ 16.15 kPa) for more than 6 years. All animals were maintained under standard conditions (21±1°C, 12-hour light-dark cycle, lights on at 06.00 h). Pelleted food and tap water were available *ad libitum*. At 09.00 h of the day of birth (PND 0), pups were moved into the hypobaric chamber as hypoxic groups. In each cage, eight pups from different litters were maintained with one lactating mother.

Definite days of age were chosen to study as they were usually correlated with important events during postnatal development in the male rat: PND 3–7 (Natal and perinatal period), PND 14 (Eye opening as well as active mylination), PND 21 (Weaning), PND 28–35 (Prepubertal days) and PND 45 (Gonadal maturation achieved).

*Hypoxia simulation*: The altitude hypoxia of 5 km (10.8% $O_2$, 54.02 kPa, $pO_2$ 11.38 kPa) was simulated in a hypobaric chamber, the local altitude (as mentioned above) as control.

*Plasma preparation and testosterone extraction*: The animals were sacrificed by decapitation between 09.00 and 10.00 h at PND 3, 7, 14, 21, 28, 35, 45 respectively. The trunk blood was collected individually in heparinized tubes and centrifuged at 3000g × 15 min at 4°C. Plasma was stored in a freezer at –35°C. After the animals were sacrificed, the bilateral testes were immediately removed, weighed and decapsulated successively, frozen in liquid $N_2$ and stored at –35°C for T extraction. T in the testes was extracted as previously described with small modification [15]. In brief, testes were thawed and 2 ml ethyl acetate was added. The tissue of the testes was homogenized by sonication. After centrifugation at 4000g × 5 min at 4°C, the ethyl acetate phase was removed and kept. The sediment was suspended with another 2 ml ethyl acetate, and was homogenized again. After centrifugation, these ethyl acetate phases were combined and dried.

*RIA*: RIA was used for the T measurement. In groups of PND 3 and PND 7, plasma was pooled from 4 pups and 2 pups, respectively, in order to obtain sufficient volume of the plasma for RIA. For other age groups, the plasma T was measured individually. RIA kits for T were purchased from China Institute of Atomic Energy (Beijing, IMK-457-9712). The measurement was processed following the instruction.

*Light microscopy and electron microscopy*: Animals were anesthetized by sodium pentobarbital (40 mg/kg BW, ip). Then the testes were fixed by whole-body perfusion with 2.5% glutaraldehyde in PBS (pH 7.2, 4°C). One testis was processed routinely with paraffin embedding for histological sections (6 μm). After H-E staining, the sections were observed using an Olympus BH-2 microscope (Japan). The contralateral testis was decapsulated, cut into ~1 mm cubes, immersed successively in 2.5% glutaraldehyde in PBS (pH 7.2, 4°C) for 1.5 h, in 1% osmium tetroxide in PBS (pH 7.2, 4°C) for 1.5 h, then rinsed and dehydrated gradually in diluted ethanol. After rinse in propylene oxide for 2 × 20 min, the blocks were infiltrated and embedded in Shell Epon812. Sections were cut on a Porter-Blum microtone with a glass knife, placed on grids and observed using a Hitachi H-800 Transmission Electron Microscope (Japan).

*Statistics*: All data were examined by one-way ANOVA followed by Student's *t*-test. A value of $p<0.05$ was considered statistically significant.

## Results

*Body weight and testes weight*

In both hypoxia and control groups, body weight and testes weight were increased with age. In the hypoxic rats, for all groups of age, the body weight was significantly ($p<0.05$ at PND 3; $p<0.01$ at PND 7 to PND 45) lower than that of age-matched control. The testes weight in all hypoxia groups except for PND 3 was significantly lower than that in control ($p<0.01$) (Figure1). At PND 45, the mean body weight in hypoxic rats was 40.84% of the control, the mean testes weight in hypoxic rats was only 22.69% of the control.

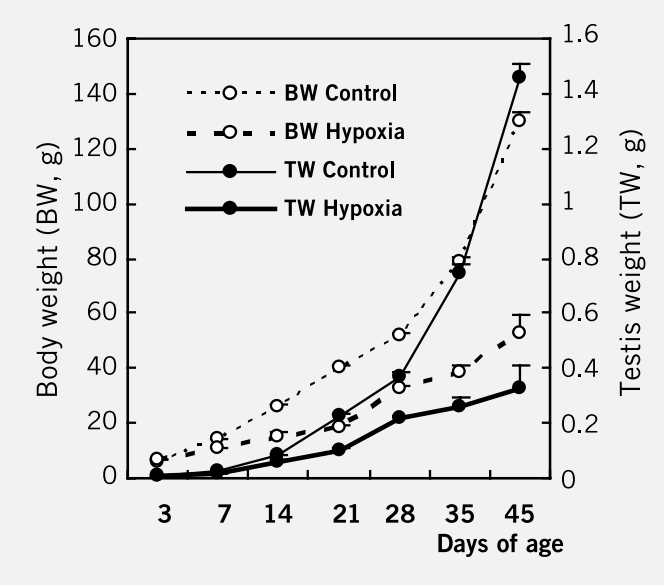


**Figure 1.** During the development in the hypoxia of 5000 m altitude, the body weight (BW) gain and testes weight (TW) increase were suppressed. Values were means ± SEM (n=7–8); $p<0.05$ in BW at PND 3, $p<0.01$ in all the groups from PND 7 to 45, versus age-matched control group.

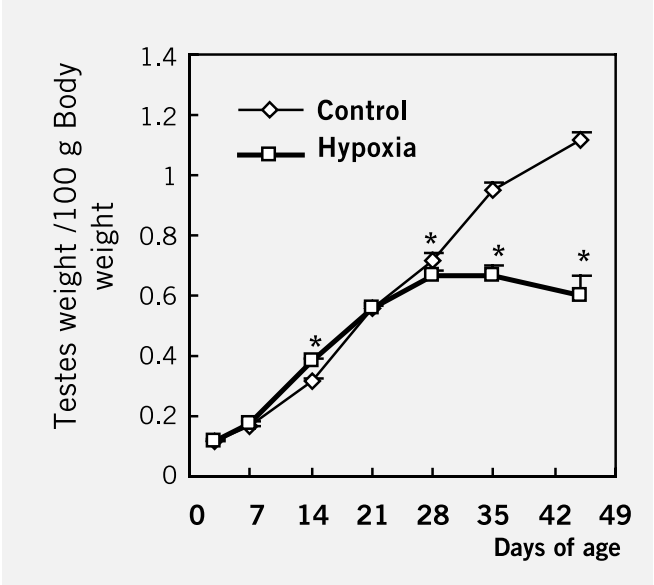


**Figure 2.** The relative testes weight (testes weight per 100 g body weight) during the development showed that hypoxia significantly arrested the testis growth after PND 28. Values were means ± SEM (n=7–8); *$p<0.05$ versus age-matched control group.

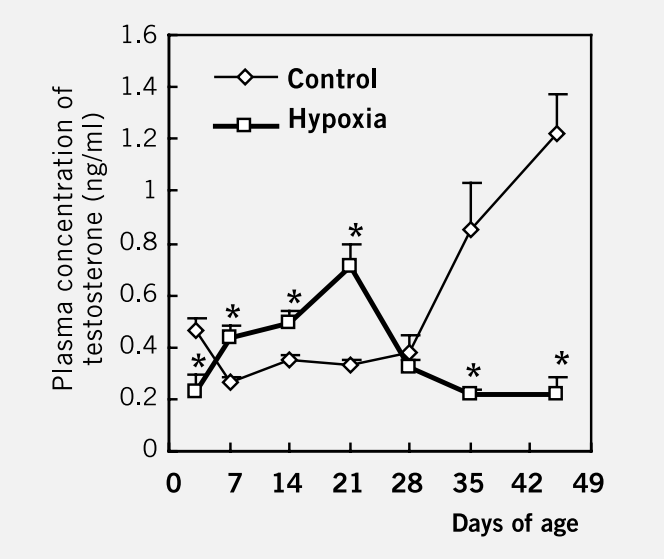


**Figure 3.** Effects of hypoxia on the plasma concentrations of T during neonatal development in rats. Values were means ± SEM (n=7–8); *$p<0.05$ versus age-matched control group.

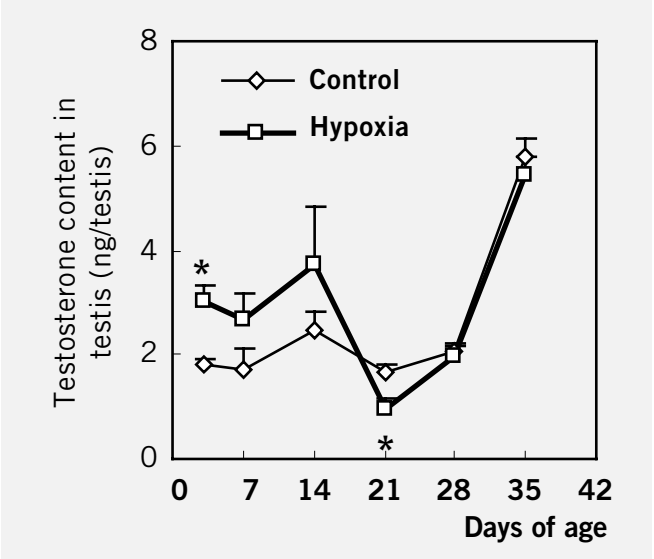


**Figure 4.** Effects of hypoxia on the T contents in the testis during neonatal development in rats. Values were means ± SEM (n=7–8); *$p<0.05$ versus age-matched control group.

The changing of the relative testes weight (testes weight / 100g body weight) with age in hypoxic rats was different from that in controls. At PND 14, the relative testes weight in hypoxic rats was significantly higher than in controls ($p<0.05$). At PND 28, 35 and 45, the relative testes weight was significantly lower than in age-matched controls ($p<0.05$). In hypoxic rats, the developing curve of relative testes weight reached like a "platform" between PND 28 and 35, then tended to decline (Figure 2).

*Testosterone concentration in plasma*

In control, the plasma T concentration remained low at PND 7, 14, 21 and 28, and then increased rapidly after PND 28. In contrast, in hypoxic rats, plasma T concentration was significantly high at PND 7, 14 and 21 (peak point), and then dramatically dropped down to a lower level after PND 21, compared with that in age-matched controls (Figure 3).

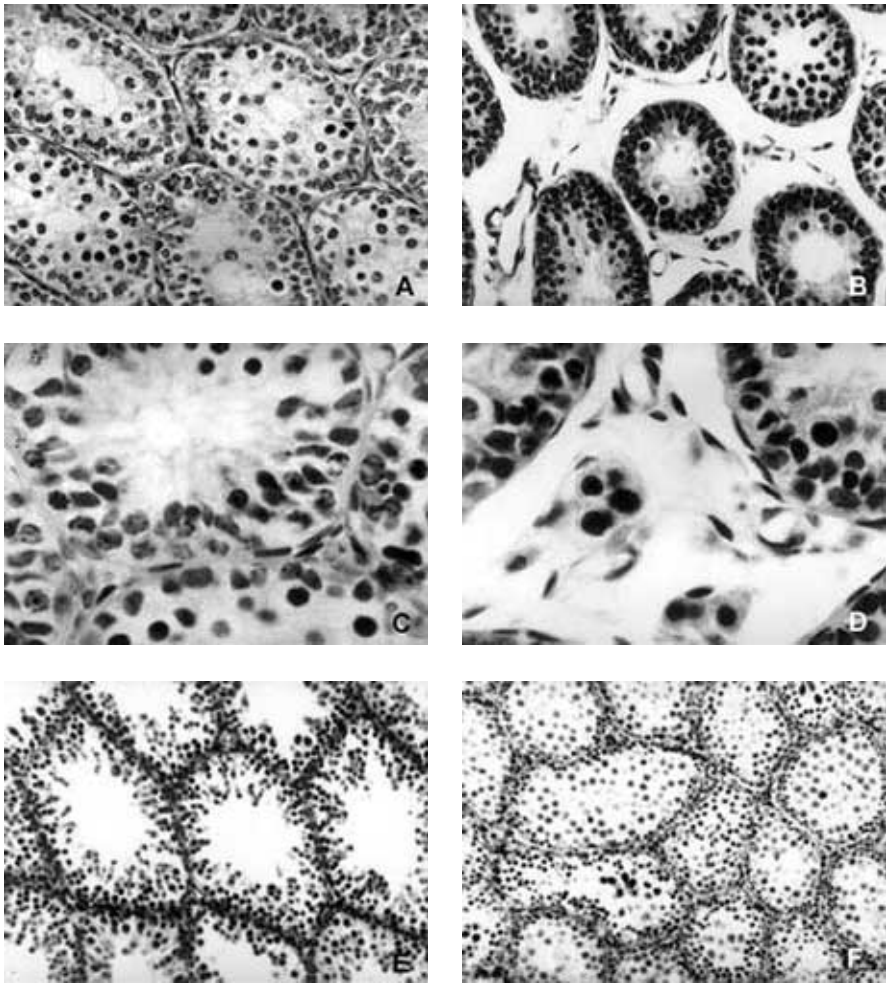


**Figure 5.** Light micrographs of the rat testis at PND 14 (A and B, 100×), PND 21 (C and D, 100×) and PND 35 (E and F, 20×) respectively, for control groups (A, C, D) and for hypoxia groups (B, D, F). In the testis of hypoxic rats, the swelling of the interstitial space and the increased vascularity in the interstitium were observed (B, D). Clusters of Leydig cells were found in the interstitial space in the testis of hypoxic rats at PND 21 (D).

*Testosterone content in the testis*

Compared with the age-matched controls, the T content in the testis in hypoxic rats was significantly increased at PND 3 ($p<0.05$), remained high at PND 7 and 14, then dropped dramatically to the lowest point at PND 21 ($p<0.05$); from PND 21, T levels increased rapidly, but there was no significant difference between controls and hypoxic groups (Figure 4).

*Histological structure in the testis*

The swelling of interstitial space was observed in the hypoxic rats at as early as PND 3. At PND 35, the swelling was ameliorated, but the tubules were still smaller in size (F). Increased vascularity was observed in the testis of hypoxic rats after PND 14 (B, D). Leydig cells containing lipid droplets were frequently found in interstitial space at PND 14 and 21 in the hypoxic rats (D). Under the light microscope, the lipid droplets appeared to be empty spaces, because of the lipid lost in processing of the tissues (Figure 5).

*Cellular ultrastructure in the testis*

In hypoxic rats at PND 21 and 28, numerous of enlarged mitochondria with distorted cristae were observed in spermatogenesis cells (H) and Leydig cells (J) (Figure 6).

## Discussion

In this study, we observed that hypoxia inhibited body weight in postnatal male rats (Figure 1), in agreement with others [1, 2, 7, 8]. Body growth inhibition during hypoxia might be related to hypoxia-induced endocrine insufficiency, especially hypothyroidism [3, 5, 6, 16]. Growth hormone (GH) is also affected by hypoxia [6]. We previously reported that chronic hypobaric hypoxia decreased GH levels in pituitary and plasma in adult rats [17]. A complementally exogenous GH could ameliorate growth retardation induced by hypoxia [18]. However, in contrast to the adult rats, hypoxia did not affect plasma GH levels in developing rats from birth to PND 7 [19, 20]. Thus in neonatal rats, the GH insufficiency seemed to be correlated with animal age and hypoxic duration. In addition to insufficiency of thyroid hormone and GH, decreased food intake seemed to be one of the reasons for the growth retardation [7]. The reduction of food intake was observed in weanling rats maintained in the hypoxia [8]. This reduced food intake in adult rats during hypoxia was ameliorated by exogenous GH administration (unpublished data). Before weaning, the growth of the pups was dependent upon the mother's lactation. We thus postulated that before the day of weaning, the arrested body growth of pups was probably influenced indirectly by the reduced food intake of the hypoxic

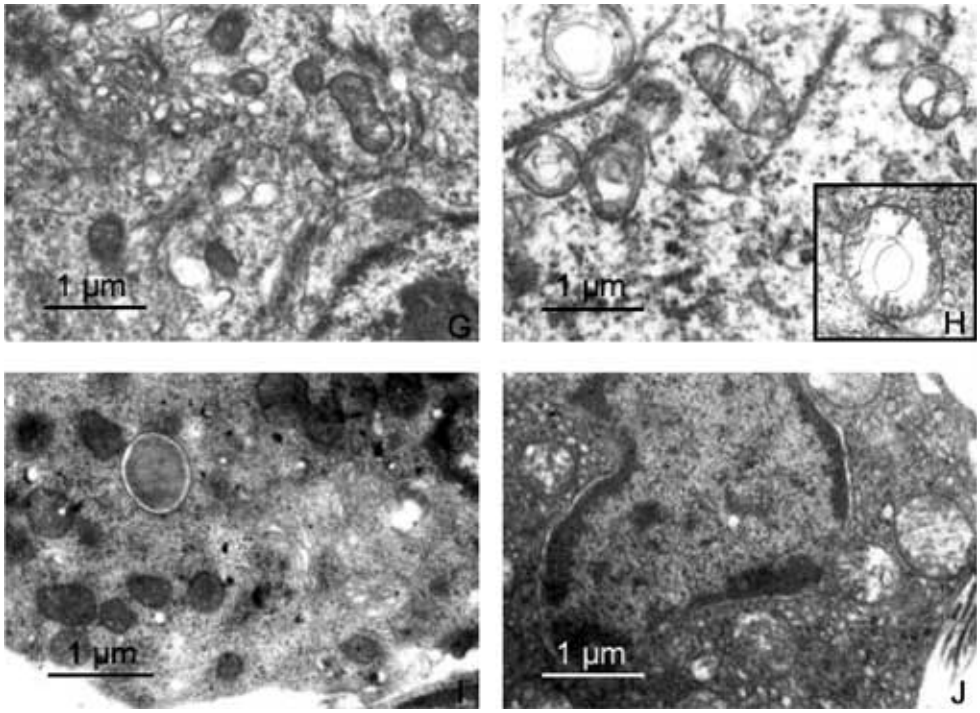


**Figure 6.** Electron micrographs of the rat testis (15000×). The enlargement of mitochondria was observed in the testis of hypoxic rats. (G) A spermatogenesis cell in the testis of control rats at PND 21; (H) A spermatogenesis cell in the testis of hypoxic rats at PND 21; An enlarged mitochondrion with distorted cristae was shown at the right bottom; (I) A Leydig cell in the testis of control rats at PND 28; (J) A Leydig cell in the testis of hypoxic rats at PND 28, showing enlarged mitochondria.

mother resulting in insufficient lactation. In the current study, we found that after PND 28 the growth velocity of the testis was severely reduced in hypoxia (Figure 2). We suggested that the hypothyroidism in early development (prior to PND 28) might be crucial for the arrested testis development during hypoxia. This suggestion was indirectly supported by the recent findings that triiodothyronine receptor is abundantly expressed in rat testis from fetal until prepuberal period [21], and its transcriptional activity and binding capacity decreased gradually from birth to adulthood [22]. In addition to thyroid hormone, GH might be important for the neonatal development of the testis in view of the presence of immunoreactive GH receptor in the rat testis at PND 10 [23].

The plasma T concentration in control rats was maintained at a low level from birth to PND 21, and began to increase after PND 28. The trend of plasma T level was due to the apoptosis of the fetal-type Leydig cells and the differentiation of adult-type Leydig cells during the neonatal period [24–27]. In hypoxic rats, T secretion was inhibited at PND 3. It was stimulated between PND 7 and 21, and followed by suppression after PND 21. From PND 3 to 14, the T content in the testis was higher than control (significant at PND 3), indicating an increased synthesis of T. The low content of T in the testis at PND 21 might represent a hypersecretion of T. From these data we assumed the days between PND 7 and 21 as a "hyper-responsive period" for T secretion in the hypoxia. Coincidently, this period partly over-lapped the "stress hyporesponsive period (SHRP)" of the hypothalamic-pituitary-adrenal (HPA) axis, which was between PND 4 and 14 in rats [28]. Besides, we had reported previously that the plasma corticosterone did not respond to hypoxia (of 5 km altitude) stress at PND 21 [29]. Nevertheless, further clarification was necessary. Interestingly, in hypoxic rats after PND 21, the plasma T level dramatically declined to the baseline, while it increased rapidly after PND 28 in controls. One of the possible explanations for this difference might be that the hypoxia arrested differentiation of adult-type Leydig cells within critical period for neonatal development through the hypoxia-induced hypothyroidism. The critical role of thyroid hormone to initiate the onset of mesenchymal cells differentiation into adult Leydig cells had been proposed by other authors [30]. It was demonstrated that neonatal hypothyroidism arrested the differentiation of adult-type Leydig cells [31, 32], and also induced a chronic reduction in circulating gonadotropins [33].

In this study, we found that the hypoxia induced morphological changes in the testis, including the interstitial swelling and mitochondria enlargement. The swelling of testis interstitium seemed to be also induced in adult male rats by chronic hypoxia, as we previously reported [34]. The mechanism of this morphological change remains unknown; however it may be related to the hypoxia-induced functional impairment of the blood-testis barrier and active substance transport in Sertoli cells [9]. As reported by other authors, hypoxia-induced enlargement of mitochondria was also observed in Sertoli cells in chicks [35] and cells of adrenal zona glomerulosa, corneal endothelium, cerebral cortex and other tissues in rats [36–38]. Mitochondria are crucial for the cell to survive hypoxia [39]. Moreover, damaged mitochondria are associated with cell damage and apoptosis [40, 41]. The above morphological observation indicated that hypoxia induced structural alterations in the testis. At PND 35 and 45, the interstitium swelling seemed to be partly amelio-

rated. Moreover, as found in other tissues in chronic hypoxia [42,43], the increased vascularity was observed in the testis interstitium of hypoxic rats except for PND 3 and 7. This increased vascularity may improve the oxygen supply to the testis, and thus represent a signal for the tissue adaptation to the hypoxia. In additional, the increased vascularity might be partly due to the increased expression of vascular endothelial growth factor in Sertoli cells, as reported recently [44].

Though after PND 28 the histological destruction was partly ameliorated, the testis growing and endocrine function of the testis was not essentially amended because: 1) after PND 28, the testes weight failed to return to the normal scale; 2) the prepuberal plasma T level was absent. Moreover, we postulated that there was a "critical period" crucial for the testis development during hypoxia in the postnatal life of rats. This "critical period" was probably within the first four weeks after birth. This was coincident with our previous finding that PNDs 25–28 were crucial for rat pituitary development in hypoxia [45,46]. In addition, whether the functional suppression and morphological change of the testis were reversible if the hypoxia were withdrawn remained to be clarified.

In summary, the prolonged hypoxia produced the retardation of the postnatal development of the rat testis, possibly because of:

1) The suppression on body growth;
2) The alteration of the testis endocrine function;
3) The structural changes in the testis.

**Acknowledgements**

This work was supported by the grant from NSFC (No. 30070289). The authors were grateful to Dr. Yang Sheng-Mei for her assistance on electron microscopy and Dr. P.S. Timiras, from the Department of Molecular and Cell Biology at the University of California, Berkeley for her some editorial improvements.

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