

Hypoxia-induced apoptosis in the bilateral testes of rats with left-sided varicocele: A new way to think about varicocele

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Abstract

Although varicocele has been studied for many years, its pathophysiology remains unclear. In this study, we investigated the expression of the α subunit of hypoxia-inducible factor 1 (HIF-1 α) and determined the apoptosis index (AI) in the testes of rats with varicoceles to study the mechanism by which varicoceles induce infertility. A total of 45 Wistar rats were divided into three groups: the control group, the sham surgery group, and the experimental group. Forty-nine days after the day of the initial partial ligation of left renal vein, all of the rats underwent orchiectomy. HIF-1 α expression in each testis was analyzed using immunohistochemical methods and ELISA. The degree of apoptosis within each testicle was determined using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). HIF-1 α immunoreactivity in the testes of the experimental group was significantly higher than that in those of the control group ($P<0.05$) or the sham group ($P<0.05$). The AI of the germ cells of rats in the experimental group was significantly higher than that in of germ cells of rats in the sham group ($P<0.001$) or the control group ($P<0.001$). Additionally, there was a significant positive correlation observed between the AI of germ cells and relative intensity of HIF-1 α in the left testis ($r=0.631$, $P=0.028$) and right testis ($r=0.707$, $P=0.01$) of rats in the experimental group. The results of this study showed that a left-sided varicocele could cause bilateral testicular hypoxia and increased germ cell apoptosis, both of which play an important role in testicular dysfunction. Furthermore, HIF-1 α is a useful factor that can be used to predict the degree of germ cell apoptosis in rat testes.

Key words: *Infertility, spermatogenesis, testis, varicocele, hypoxia-inducible factor*

Introduction

Although varicocele is a physical abnormality present in 2-22% of the adult male population, we still do not understand the exact relationship between reductions in male fertility and varicocele (Dohle G R et al, 2005). This explains why nearly two-thirds of men with varicoceles remain fertile, and why controversy continues to surround the clinical utility of varicocele treatment (Sandlow J I, 2004). Although the fact that many hypotheses have been proposed in previous studies to explain the pathophysiology underlying the relationship between varicocele and male infertility, including endocrine and testicular paracrine

imbalances (Skoog S J et al., 1997), hyperthermia (Wright E J et al., 1997, Hjollund N H et al., 2002), hypoxia (Kilinc F et al., 2004), apoptosis (Barqawi A et al., 2004), and retrograde flow of adrenal blood (Mobley D F, 1974), none can independently explain this phenomenon.

However, it seems that many of these hypotheses are related to apoptosis. In other words, many varicocele-associated factors can affect testicular function by inducing germ cell apoptosis, such as hyperthermia with resultant apoptosis (Wright E J, et al., 1997, Hjollund N H et al., 2002, Lue Y H et al., 1999, Yin Y et al., 1998) and endocrine and testicular paracrine imbalances leading to apoptosis (Skoog S J et al., 1997, Yin Y et al., 1998). Previous histopathological studies have revealed stagnation of blood flow and degenerative changes in testes with varicocele, which were attributed to lack of adequate oxygenation in these testes (Lee J D et al., 2006, Kilinc F et al., 2004). However, information regarding the relationship between hypoxia and germ cell apoptosis in rats with varicocele rat is lacking. Furthermore, although varicocele is considered to be a predominantly unilateral (left-sided) disease, bilateral damage is caused by unilateral varicoceles. Therefore, we hypothesized that bilateral testicular hypoxia induced by left-sided varicoceles could lead to bilateral germ cell apoptosis.

To test this hypothesis, we sought to determine the presence or absence of bilateral testicular hypoxia by detecting the expression of HIF-1 α , which is an intrinsic marker for tissue hypoxia (Vukovic V et al., 2001), and germ cell apoptosis in the bilateral testes of rats with left-sided varicoceles. We also sought to determine the relationship between germ cell apoptosis and hypoxia.

Materials and methods

Forty-five male Wistar rats (purchased from the Center for New Drug Evaluation of Shandong University) weighing 250-300 g were used in this study. All animals were fed the same diet and maintained in a constant environment with a 12:12-hour light dark cycle. We maintained an antiseptic environment during our surgical procedures. General anesthesia was induced via inhalation of ether and intraperitoneal injection of 100 mg ketamine. The rats were randomly divided into three groups: the experimental group [comprised of 15 rats with varicocele whose left renal veins were partially ligated using the method described by Turner (Turner T T, 2001)]; the sham operation group (comprised of 15

rats that underwent a sham procedure, i.e., the same operation performed on the experimental group, except the ligatures were only placed in position and not tied down); and the control group (comprised of 15 control rats that did not undergo initial surgery). A successful varicocele rat model were required to have a spermatic vein with a diameter >1.0 mm with visible varicosity at the distal end of the spermatic vein. Three rats in experimental group, two in the sham operation group, and three in control group, were excluded for unsuccessful surgery, death, or the presence of a palpable spermatic vein. All rats underwent orchiectomy 49 days after the day of the initial surgery.

Quantitative analysis of HIF-1 α expression. HIF-1 α levels were measured by sandwich ELISA using a Protein Detector ELISA Kit (BLE110-125, Bethyl Laboratories, Montgomery, AL, USA) according to the manufacturer's instructions. The plate, which was composed of 96 wells, was incubated for 1 h at RT with anti-HIF-1 α antibody (1:250, clone MAb, IMG-629, IMGENEX, San Diego, CA, USA). Each testis sample (0.03 g) was mixed with 0.4 ml Mammalian Tissue protein extraction reagent (Shennengbocai, Shanghai, China) and 4 μ L proteinase inhibitor (10 mg antipain, 5 mg leupetin, and 50 mg benzamidine dissolved in 5 ml aprotinin), then homogenized on ice. After washing the plate, the homogenates were incubated with the anti-HIF-1 α antibody (1:750, clone PAb, sc-10790, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After incubation with these primary antibodies, anti-rabbit IgG and peroxidase-linked species-specific fragments (1:10000, NA 9340, Amersham Pharmacia Biotech, Amersham, UK) were added to each well and incubated for 1 h. Then, the enzymatic reaction was carried out at RT for 15 min using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid). The absorbance at 405 nm was measured using a spectrophotometer (Tianjin Tuopu Instrument company, Tianjin, China).

Immunohistochemical analysis of HIF-1 α expression. Immunohistochemical staining was performed with an SP kit (Zhongshan, Beijing, China) using the standard avidin-biotin-peroxidase complex technique. Sections from each testicular specimen were briefly deparaffinized, dehydrated, and immersed in 10mM sodium citrate buffer (pH 6.0). To detect hypoxia-inducible factor (HIF-1 α), the sections were heated in a microwave at a temperature of 95°C for 10 min. Endogenous peroxidase was then inactivated by incubating the sections with 3% hydrogen peroxide, and nonspecific reactions were blocked by incubating sections with a solution containing 10% normal goat serum. The sections were then

incubated with the primary antibody overnight at 4°C. The primary antibody was mouse monoclonal HIF-1 α IgG antibody (1:1000, Abcam Company, MA, USA). After incubation with the antibody, the sections were incubated for 15 min at 37°C with biotinylated anti-mouse immunoglobulins and then with streptavidin-conjugated horseradish peroxidase. Between and after the two steps, the slides were rinsed three times with phosphate-buffered saline (PBS) for 15 min. The specimens were then incubated with diaminobenzidine and hydrogen peroxide. Negative controls were processed at the same time. The results of the immunohistochemical analysis of the HIF-1 α protein were classified as follows: -, which means there are no staining cells in the specimen; and “+” means nuclear staining cells are less than 1% of all the cells; ++, nuclear staining in 1–10% of cells with/without distinct cytoplasmic staining; and +++, nuclear staining in more than 10% of cells with/without distinct cytoplasmic staining (Zhong H, et al).

Analysis of apoptosis. In each testicular specimen, we assessed the degree of apoptosis that was present using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). These analyses were performed with an ApopTag in situ detection kit (Intergen Company, NY, USA). Paraffin-embedded tissue specimens were cut into 6- μ m sections and mounted on microscope slides. The slides were deparaffinized using xylene and decreasing concentrations of ethyl alcohol, and washed in 0.01 M PBS (pH 7.4). Endogenous peroxidase was inactivated by immersing the sections in 3% hydrogen peroxide in methanol for 10 min at room temperature. The sections were then incubated with 20 μ g/ml proteinase K for 30 min at 37°C and staining was performed according to the manufacturer’s (Intergen Company, NY, USA) instructions. Sections were briefly washed with phosphate-buffered saline for 10 min. TdT marking reaction solution were added to cover each section, and the slides were incubated in a humidified chamber at 37°C for 90 min. The reaction was stopped by washing the slides in PVS for 10 min. Finally, the slides were incubated with diaminobenzidine and hydrogen peroxide and counterstained with hematoxylin. Negative controls were processed at the same time. We determined the AI to record the presence or absence of apoptosis in each tissue specimen. AI was calculated as the number of positively stained cells detected in five randomly selected fields at 400x magnification divided by the total number of cells in those five fields.

Statistical Analysis

SPSS 11.5 for Windows (SPSS, Chicago, IL, USA) was used for statistical analysis.

The data in Table 1 and Table 2 were analyzed using descriptive analyses, one-way analysis of variance (ANOVA), and Fisher's exact test. To examine the correlation between the AI of the germ cells and the relative intensity of HIF-1 α staining, Spearman rank correlation coefficients were calculated. $P < 0.05$ was defined as the level of statistical significance.

Results

As shown in Table 1 and Figure 2, HIF-1 α immunoreactivity (present in 33 of 37, or 89.19%, of rat testes) was observed in almost every rat testis in the three groups, indicating that there was basal production of HIF-1 α during normal spermatogenesis. The frequency of HIF-1 α positivity (Table 1, Figure 1) in the left testes of rats in the experimental group was not significantly higher than the rate of HIF-1 α positivity in the left testes of rats in the control group ($P = 0.478$) or sham group ($P = 1.000$). However, the frequency of strong staining (++ or +++) was significantly higher in the experimental group than in the control group ($P < 0.001$) and the sham group ($P < 0.001$). The expression of HIF-1 α (as detected by ELISA, Table 2) in the experimental group was significantly higher than expression levels in the control group ($P = 0.009$) or the sham group ($P = 0.005$), and there was no significant difference between HIF-1 α expression levels in the control and the sham group ($P = 0.690$). The right testes of rats in the experimental group had significantly higher AI to the left sided testes of rats in the control ($P = 0.012$) and sham group ($P = 0.004$). Additionally, there were no significant differences observed between the left and right testes of experimental group in HIF-1 α expression ($P = 0.445$) or in the frequency of strong HIF-1 α positivity ($P = 1.000$). It was also evident that there were fewer cells in each spermatid tubule of rats in the experimental group than rats in the control or sham group, and that the testicular texture of rats in the experimental group was less firm than that of rats in the other two groups (Figure 1).

The AI of germ cells in the experimental group (both left and right, 18.8 ± 7.2) was significantly higher than that of germ cells in the sham group (0.22 ± 0.57 , $P < 0.001$) and control group (1.28 ± 3.5 , $P < 0.001$), and there were no significant differences observed between the control group and the sham group

($P=0.303$). The left testes (19.09 ± 0.061) had similar AI values as the right testes (18.54 ± 8.4) among rats in the experimental group ($P=0.854$).

Figure 2 shows that the correlation between the AI of the germ cells and the HIF-1 α detected by ELISA in the experimental group was significantly positive (Left: $r=0.631$, $P=0.028$; Right: $r=0.707$, $P=0.01$).

Figure 3 provides some information regarding the association between HIF-1 α expression in the left and right testes of experimental group ($r=0.72$, $P<0.001$) as well as the association between the AI of the germ cells in the left and right testes ($r=0.968$, $P<0.001$).

Discussion

Varicocele is a recognized cause of male infertility that affects 25-40% of patients with abnormal semen analyses (Dohle G R et al., 2005). Because the current information regarding varicocele pathophysiology does not apply to all clinical situations and because varicocele patients are not treated etiologically, it is baffling why some men with varicoceles have normal semen and can father children, while others fail to regain fertility despite corrective surgery (Ku J H et al., 2005). Therefore, the identification of the mechanism by which varicocele induces infertility is a crucial step in treating this pathology. Previous studies have shown that varicoceles may induce testicular hypoxia. Lee, Jeng, and Lee demonstrated that hypoxia-related pathophysiological changes occurred in response to conditions of hypoxic stress in the internal spermatic veins of varicocele patients, suggesting the occurrence of hypoxia in the testicular tissue (Lee J D et al., 2006). A previous study obtained similar results using an animal model of varicocele (Kilinc F et al., 2004). Additionally, Wang's study showed that epididymal dysfunction had some relationship to hypoxia induced by varicocele (Wang HF et al., 2008). Thus, testicular hypoxia is an important pathophysiological characteristic of patients with varicocele, and may contribute to varicocele-related male infertility. The hypoxia is thought to be caused by increased hydrostatic pressure in the venous drainage system, which exceeds the pressures in the testicular arterial microcirculation due to venous stasis produced in the disease (Gat Y et al., 2006). However, all previous studies have focused on unilateral testicular hypoxia. Thus, no studies have previously examined whether or not there is right testicular hypoxia in patients with left-sided varicoceles. Using venography, Gat et al. found that

varicocele was a bilateral vascular disease, and that right-sided venous return problem was affecting 86% of infertile men with clinically evident left-side varicoceles. (Gat Y et al., 2006). However, no previous studies have identified the mechanism underlying bilateral testicular dysfunction in patients with unilateral varicocele. In our study, higher levels of HIF-1 α expression, which is an intrinsic marker of tissue hypoxia (Vukovic V et al., 2001), were detected in the right and left testes of rats with left-sided varicoceles than sham and control rats (Table 1, Figure 1, Table 2). Additionally, HIF-1 α expression in the right and left testes of rats in the experimental group did not differ.

Apoptosis, also called programmed cell death, is a normal process that occurs during spermatogenesis. It can affect different steps of spermatogenesis, first during puberty at the beginning of spermatogenesis, and later in adult testicles, by regulating normal spermatogenesis (Haouzi D, 2008). About 75% of all preleptotene spermatocytes are destroyed via apoptosis (Huckins C, 1978), which contributed to the elimination of spermatogenic cells in this study. Early studies found that apoptosis was decreased in the testes of men with varicoceles, and these studies found no significant correlation either between the AI and the sperm concentration on seminogram or the AI and the mean Johnsen's scores of these patients (Fujisawa M et al., 1999). However, most current studies indicate that abnormally high levels of germ cell apoptosis may contribute to testicular failure and male infertility in varicocele patients. Benoff et al. documented a higher percentage of apoptotic nuclei within the seminiferous epithelium of varicocele patients than within the seminiferous epithelium of control patients. These authors also documented a high degree of concordance of apoptotic scores between left and right testicular biopsies from the same varicocele patient (Benoff S H et al., 2004). Similar results have been obtained using a rat model of varicocele (Barqawi A et al., 2004). Furthermore, Chen et al. demonstrated the existence of increased apoptosis in the ejaculated spermatozoa in patients with varicocele (Chen C H et al., 2004). Our study reports that the AI values of the germ cells of rats in the experimental group were higher than the AI values of rats in the sham or control groups (Figure 1 and Table 2). However, it remains unclear how these sperm-related abnormalities are associated with varicocele. Therefore, we performed this study in an attempt to determine the mechanism by which varicocele induces germ cell apoptosis.

HIF-1 α is constitutively expressed and stabilized in the testis, where it may play a role in testicular

homeostasis. Additionally, a previous study found that it may affect male reproductive function by regulating Hsd3b1 transcription (Lysiak JJ et al., 2009). Under hypoxic conditions, stabilized HIF-1 α can activate the transcription of several genes, including angiogenic factors (vascular endothelial growth-factor), growth factors (insulin-like growth-factor- II), glucose transporters (GLUT-1), and glycolytic enzymes (Aldolase A/C). Thus, HIF-1 α appears to play an important role in protecting solid tumors from hypoxia by promoting angiogenesis, inducing the expression of growth factors, preventing apoptosis, or increasing anaerobic metabolism (Kilic M et al., 2007). Kilinc et al. documented increased VEGF expression, angiogenesis, and HIF-1 α in the testicular tissue of rats with varicoceles (Kilinc F et al., 2004). This means that HIF-1 α is beneficial for tissue survival under hypoxic conditions, which are induced by the presence of a varicocele. However, our study showed that there was a positive correlation between the AI of germ cells and the relative intensity of HIF-1 α staining in the bilateral testes of the rats in the experimental group (Figure 2). This indicated that, under conditions of testicular hypoxia induced by varicocele, HIF-1 α promoted germ cell apoptosis. Carmeliet's study adds weight to our results. They found that hypoxia and hypoglycemia reduced proliferation and increased apoptosis in rHIF-1 $\alpha^{+/+}$ embryonic stem (ES) cells, but not in ES cells with inactivated HIF-1 α genes (HIF-1 $\alpha^{-/-}$) (Carmeliet P et al., 1998). Similar results were obtained by Manfred and Reet in non-small cell lung cancer; HIF-1 α expression was found to be positively correlated with the apoptotic rate of tumor cells and the presence of pro-apoptotic factors including caspase-3, Fas, and Fas ligand. (Manfred V and Reet K, 2000). Thus, HIF-1 α can promote both cell survival and apoptosis under hypoxic conditions, and the dominant effect that is exerted by HIF-1 α depends on the type of tissues and the degree of hypoxia. Our study showed that, under the hypoxic conditions induced by varicocele in testicular tissue, HIF-1 α 's primary effect was harmful rather than beneficial, as it was found to be associated with increased rates of apoptosis. We also found that left-sided varicoceles can induce bilateral testicular hypoxia, which can cause increased germ cell apoptosis in the bilateral testes. In addition, reactive oxygen species (ROS), which can be created during an intermediate event between hypoxia and the induction of apoptosis, also play an important role in apoptosis induced by hypoxia. ROS are oxygen metabolites, and include superoxide anions, hydrogen peroxide, hydroxyl radical, hydroperoxyl radical, and nitric oxide. Excess production of ROS could

damage spermatozoa by inducing oxidative stress and subsequently damaging cellular lipids, proteins, and DNA (Agarwal A et al., 2009, Mostafa T et al., 2006). Based on the positive correlation between left- and right-sided HIF-1 α expression and the left- and right-sided AI of germ cells that we observed, we confirmed that left-sided varicoceles could cause bilateral testicular damage. We also found that the degree of damage observed in the left and right testis were very similar.

In conclusion, left-sided varicoceles can induce bilateral testicular hypoxia, which can in turn cause increased germ cell apoptosis in the bilateral testes. Notably, the changes observed in both testes of rats with left-sided varicoceles were very similar. Therefore, it is possible that varicocelectomy could prevent testicular dysfunction by interrupting testicular hypoxia. The mechanism by which hypoxia induces apoptosis in the testes of rats with varicoceles, especially the role that ROS play, deserves further research.

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Figure Legends

Figure 1. HIF-1 α expression (B,D,F,H) and germ cell apoptosis (A,C,E,G) in the left testes of the experimental group (A,B), the right testes of experimental group (C,D), left testes of the sham group (E,F) and control group (G,H). Germ cell apoptosis was detected by TUNEL and HIF-1 α expression was detected using immunohistochemistry. Black arrow: apoptotic cells and HIF-1 α -positive cells (stained with DAB). All fields were viewed at a magnification of 400x.

Figure 2. The correlation between HIF-1 α expression (as detected by ELISA) and germ cell apoptosis among rats in the experimental group.

Figure 3. The correlation between HIF-1 α expression and the AI of germ cells in the left and right testes of rats with varicocele.

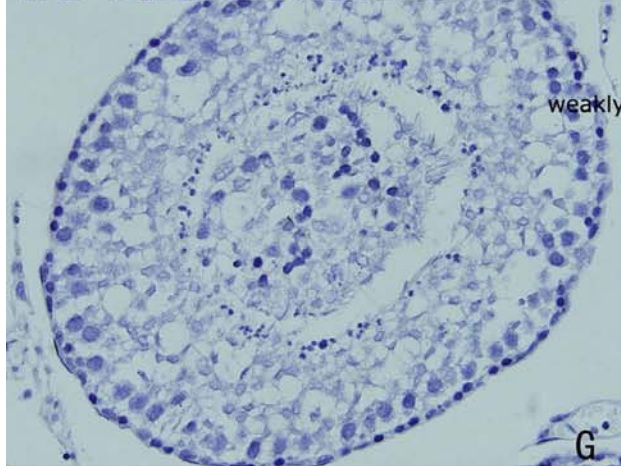
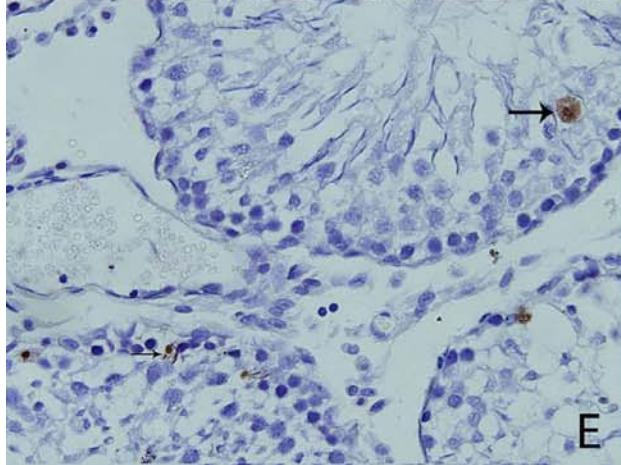
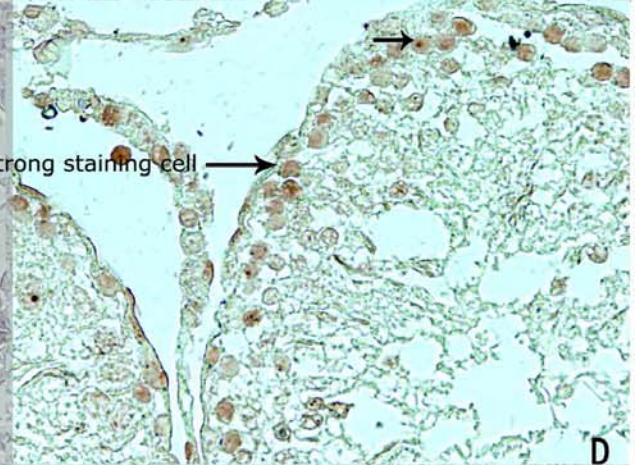
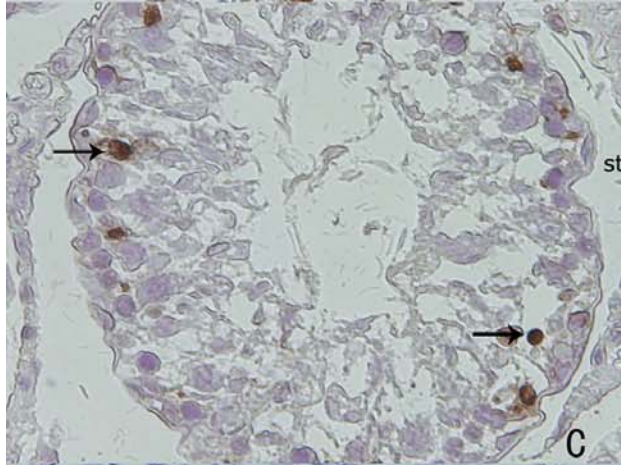
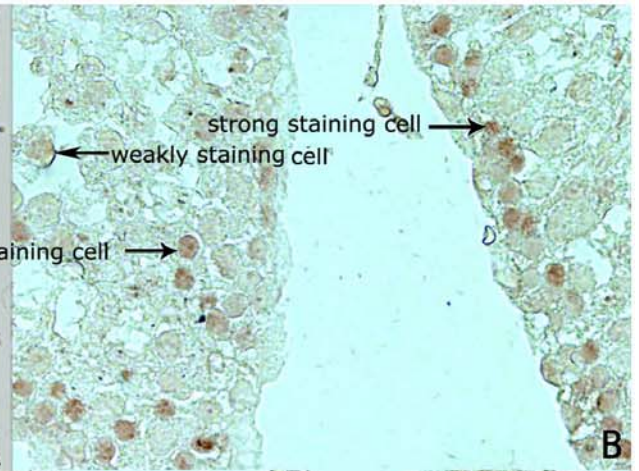
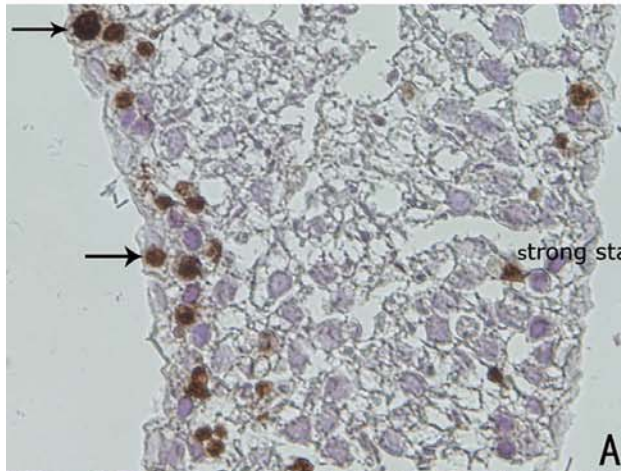
Table 1. HIF-1 α expression in the testes of rats in all three groups as detected by immunohistochemical analysis.

Group	n	-	+	++	+++	Positive (%)	Stronger than + (%)
Control	12	2	9	1	0	10 (83.33)	1 (16.67)
Sham	13	1	12	0	0	12 (92.31)	0 (0.00)
Experimental (left)	12	0	2	2	8	12 (100.00)	10 (83.33)
Experimental (right)	12	1	1	1	9	11 (91.67)	10 (83.33)

Table 2. Quantitative analyses of HIF-1 α expression (as detected by ELISA) and the apoptotic index of the testes of rats in the three groups.

	Control group	Sham group	Experimental group (left)	Experimental group (right)
No.	12	13	12	12
HIF-1 α	0.024 \pm 0.025	0.028 \pm 0.014	0.192 \pm 0.113	0.178 \pm 0.081
Apoptotic staining (n)	2	2	12	11
AI (%)	1.28 \pm 3.5	0.22 \pm 0.57	19.09 \pm 0.061	18.54 \pm 8.4

* Data are presented as means \pm SD.



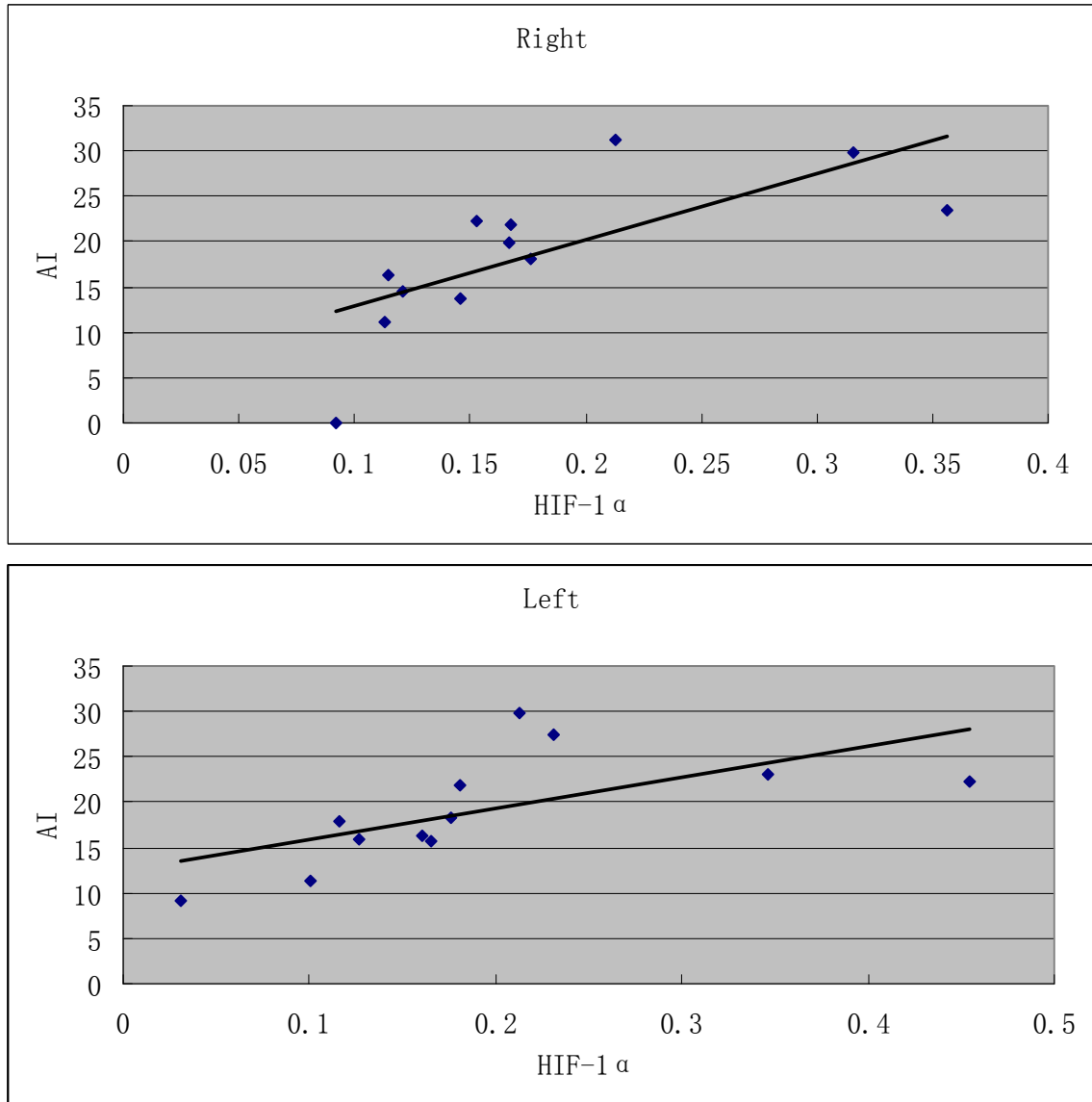


Figure 2, Wang et al.

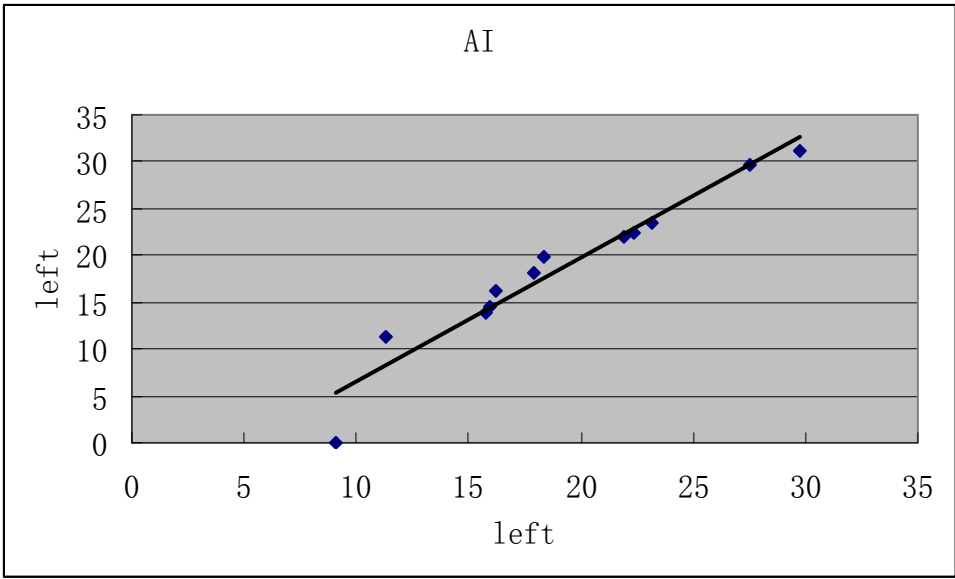
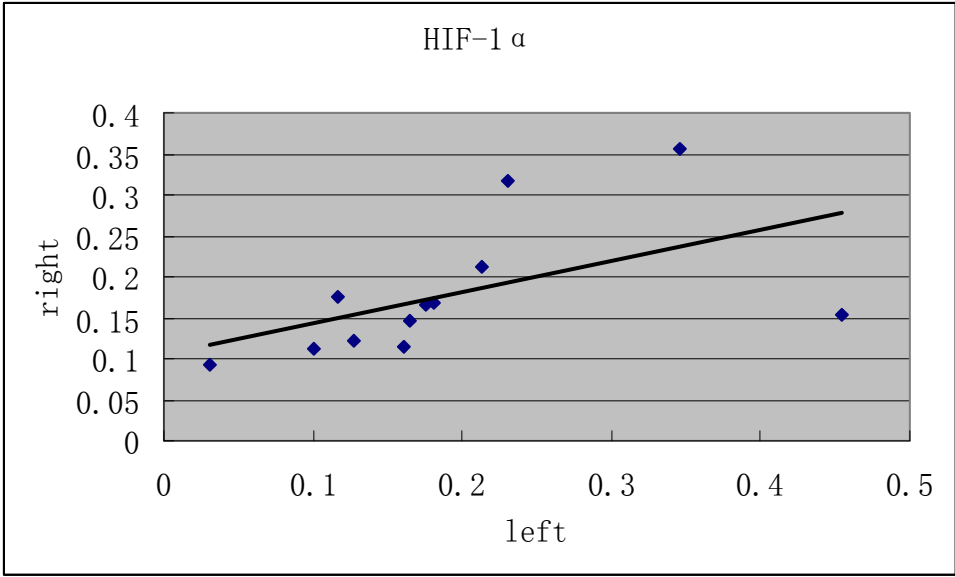


Figure 3, Wang et al.