

# Increased Expression of Hypoxia-Inducible Factor-1 $\alpha$ in the Internal Spermatic Vein of Patients With Varicocele

Jane-Dar Lee, Shaw-Yeu Jeng and Tsung-Han Lee\*

From the Department of Life Sciences (JDL), National Chung-Hsing University and Division of Urology (JDL, SYJ), Department of Surgery, Armed Forces Taichung General Hospital, Taiwan, Republic of China

**Purpose:** Varicocele is recognized as a cause of male infertility. Testis hypoxia may be one of the possible mechanisms of varicocele. We examined whether tissue hypoxia occurred in the ISV of patients with varicocele by detecting the expression of HIF-1 $\alpha$ .

**Materials and Methods:** The study group consisted of 8 patients with grade 3 left varicocele. The control group consisted of 6 volunteers with left indirect inguinal hernia. Using a left inguinal surgical incision, a 1 cm section of ISV was resected from each patient in both groups as specimens for immunoblotting and immunohistochemical staining of HIF-1 $\alpha$ . Results were analyzed using Student's t test.

**Results:** HIF-1 $\alpha$  immunoblots from both groups revealed a single band. The relative intensity of the HIF-1 $\alpha$  protein band was  $10.92 \pm 2.70$  in the control group and  $73.15 \pm 8.93$  in patients with varicocele (ie 7-fold higher).

**Conclusions:** HIF-1 $\alpha$  expression in the ISV of patients with varicocele was significantly higher than in the control group. This directly shows that hypoxia related pathophysiological changes have occurred in the ISV of patients with varicocele and that hypoxia may have also occurred in the testicular tissue. Thus, it would be of interest to investigate whether decreasing HIF-1 $\alpha$  activation and testis hypoxia could reduce the recurrence of varicocele. To our knowledge, this is the first report on HIF-1 $\alpha$  expression in human ISV. Additional studies will be necessary to clarify the relationship between testis hypoxia and male infertility in patients with varicocele.

*Key Words: hypoxia-inducible factor 1, alpha subunit, varicocele, infertility, male*

Varicocele is an engorgement and dilatation of the pampiniform plexus above the testis. Varicocele is found in 15% to 20% of men, with the left side being the most commonly affected.<sup>1,3</sup> Pampiniform plexus veins drain into the ISV on the left side, which then drains into the renal vein. Varicocele is the most common cause of male infertility in adults. Approximately 30% to 40% of men evaluated for infertility have varicocele.<sup>3-6</sup> Several hypotheses have been postulated to explain the effects of varicocele on testicles such as testis hypoxia,<sup>2</sup> scrotal hyperthermia,<sup>3</sup> retrograde flow of adrenal blood,<sup>3,4</sup> pituitary-gonadal hormonal dysfunction,<sup>3</sup> negative impact of reactive oxygen species on testicular function<sup>4,7</sup> and germ cell apoptosis.<sup>6,8</sup> Hypoxia regulates various physiological responses including vasodilatation, angiogenesis, erythropoiesis and glycolysis.<sup>9-16</sup> These physiological responses have been found in a model of varicocele in rat testes.<sup>9</sup>

Hypoxia is partially regulated by transcription factors of the hypoxia-inducible factor family. HIF-1 is considered the master regulator of the response to hypoxia.<sup>9-11</sup> Active HIF-1 is a heterodimer consisting of  $\alpha$  and  $\beta$  subunits.<sup>10-14</sup>

The activity of HIF-1 is mainly determined by HIF-1 $\alpha$ ,<sup>11-16</sup> while the HIF-1 $\beta$  subunit is constitutively produced and not regulated by oxygen tension. HIF-1 subunits are widely distributed in mammalian tissues from nearly all organisms.<sup>11-16</sup> When intracellular oxygen reaches a critically low threshold, HIF-1 $\alpha$  subunits are rapidly protected from proteasomal degradation, allowing HIF-1 $\alpha$  and HIF-1 $\beta$  subunits to associate and form an active HIF-1. Under hypoxia, HIF-1 binds to hypoxia response genes to restore oxygen homeostasis by activating hypoxia sensitive genes involved in vasodilatation, angiogenesis, erythropoiesis and glycolysis. The HIF response is one of the most important adaptive mechanisms occurring in tissues subjected to hypoxia.<sup>9-16</sup>

To determine whether hypoxia occurs in varicocele, the ISV from patients with varicocele was collected during surgery and HIF-1 $\alpha$  expression was measured. This first report analyzing HIF-1 $\alpha$  expression in human ISV samples provides new insights on the relationship between hypoxia of the testis, varicocele and male infertility.

## MATERIALS AND METHODS

**Patients and tissue samples.** The study group consisted of 8 patients (20 to 25 years old) with grade 3 left varicocele who underwent evaluation for varicocele by physical examination and color flow Doppler sonography.<sup>17-19</sup> Varicocele were graded according to Dubin and Amelar as grade 1—varicocele palpable only during the Valsalva maneuver, grade 2—varicocele palpable in standing position and grade

Submitted for publication April 12, 2005.

Supported by a grant from the Armed Forces Taichung General Hospital, Taiwan, Republic of China.

\* Correspondence: Department of Life Sciences, National Chung-Hsing University, 250 Kuo-Kuang Rd., Taichung 40227, Taiwan, Republic of China (telephone: +886 4 2285 6141; FAX: +886 4 2284 0476; e-mail: thlee@dragon.nchu.edu.tw).

See Editorial on page 818.

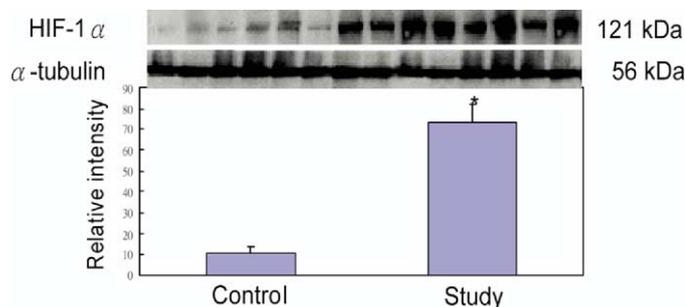


FIG. 1. Representative immunoblot and relative intensity of HIF-1 $\alpha$  protein in study (varicocele) and control (inguinal hernia) groups.  $\alpha$ -Tubulin was used as loading control. Values of HIF-1 $\alpha$  expression are normalized relative to  $\alpha$ -tubulin expression. Mean HIF-1 $\alpha$  protein in patients in study group was 7 times higher than that in control group.

3—varicocele detectable by visual scrutiny alone.<sup>17,18</sup> To prevent interobserver bias, physical examinations were performed by 1 physician. The control group consisted of 6 volunteers (20 to 25 years old) with indirect left inguinal hernia in whom varicocele was ruled out by physical examination and color flow Doppler sonography (ISV diameter less than 2 mm).<sup>19</sup> A left inguinal surgical incision was performed in all patients,<sup>20</sup> and 1 cm of ISV was resected and stored at -80C for the subsequent immunoblotting and immunohistochemical staining of HIF-1 $\alpha$ .

**Antibodies.** All the antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, California. The 2 primary antibodies used in the present study were HIF-1 $\alpha$  polyclonal antibody (sc-10790, 200  $\mu$ g/ml) and  $\alpha$ -tubulin monoclonal antibody (sc-5286, 200  $\mu$ g/ml, used as loading control). The secondary antibodies for immunoblots were AP conjugated goat anti-rabbit IgG (sc-2007, for detection of HIF-1 $\alpha$ ) or goat anti-mouse IgG (sc-2008, for detection of  $\alpha$ -tubulin). The secondary antibody for immunohistochemistry was horseradish peroxidase conjugated goat anti-rabbit IgG (sc-2004).

**HIF- $\alpha$  immunoblotting.** Each ISV sample (1 cm) stored at -80C was mixed with 0.5 ml lysis reagent (Promega, Cat. No. E1531, Madison, Wisconsin) and 5  $\mu$ l proteinase inhibitor (10 mg antipain, 5 mg leupeptin and 50 mg benzamidine dissolved in 5 ml aprotinin), then homogenized on ice. The aliquots of 100  $\mu$ g of the homogenate and pre-stained molecular weight standards (Bio-Rad, Hercules, California) were heated at 100C for 5 minutes and fractionated by electrophoresis on 10% Sodium dodecyl sulfate-polyacrylamide gel performed at 140 V for 3.5 hours. Gels were then equilibrated for 15 minutes in 25 mM Tris-HCl pH 8.3 containing 192 mM glycine and 20% (V/V) methanol. Electrophoresed proteins were transferred onto nitrocellulose (Hybond-C Extra Supported, 0.45 Micro, Amersham, Piscataway, New Jersey) using a Transphor Unit (Hoefer Scientific Instruments, San Francisco, California) at 100 mA for 14 hours. According to the manufacturer's manual, the antibodies of HIF-1 $\alpha$  and  $\alpha$ -tubulin detected molecular weights of about 135 and 50 kDa, respectively, so the transferred nitrocellulose membrane (blot) was cut into upper and lower parts at 90 kDa. The upper and lower blots were incubated separately at room temperature for 2 hours in blocking buffer and then for 3.5 hours with HIF-1 $\alpha$  antibody (upper

blot) and  $\alpha$ -tubulin antibody (lower blot) diluted 1:200 for each antibody in antibody binding buffer (100 mM Tris-HCl pH 7.5, 0.9% [W/V] NaCl, 0.1% [V/V] Tween-20 and 1% [V/V] fetal bovine serum). Blots were washed 3 times in blotting buffer, and incubated in AP conjugated goat anti-rabbit IgG (upper blot) and AP conjugated goat anti-mouse IgG (lower blot) for 1 hour (diluted 1:1000 for each antibody in binding buffer). Blots were washed 3 times in blotting buffer for 10 minutes and signals were developed with nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphate (Chemicon, Temecula, California). Immunoblots were scanned and the relative intensity of the immunoreactive bands was measured.

**Immunohistochemistry for HIF-1 $\alpha$ .** Formalin fixed and paraffin embedded sections (4  $\mu$ m) of ISV were stained with hematoxylin and eosin. For HIF-1 $\alpha$  immunohistochemistry, sections were dehydrated and immersed in 10<sup>3</sup> M sodium citrate buffer (pH 6.0). Sections were then heated at 60C for 10 minutes. An avidin-biotin-peroxidase complex was used to detect HIF-1 $\alpha$ . Endogenous peroxidase was inactivated by incubating sections with 3% hydrogen peroxide, and nonspecific reactions were blocked by incubating sections in a solution containing 5% normal horse serum and 1% normal goat serum. Sections were incubated with the primary antibody overnight at 4C. Rabbit serum was used for negative control (data not shown). HIF-1 $\alpha$  expression was assessed using peroxidase conjugated rabbit polyclonal antibody IgG (Santa Cruz Biotechnology, Santa Cruz, California, dilution 1:100). After 3 rinses with phosphate buffered saline, sections were incubated with diaminobenzidine substrate for 5 minutes. Finally, sections were rinsed with distilled water and counterstained with hematoxylin. Sections were observed using an Olympus BX50 light microscope and photographed with a Nikon CP5000 digital camera. Statistical analysis was conducted using Student's t test (SPSS<sup>®</sup> software) and significance was established at  $p < 0.05$ .

## RESULTS

The immunoblots revealed a single band of HIF-1 $\alpha$  protein (at 121 kDa) in all patients (fig. 1). The relative intensity of the HIF-1 $\alpha$  band was approximately 7-fold higher in patients with varicocele than in the control group (73.15  $\pm$  8.93 vs 10.92  $\pm$  2.70, see table and fig. 1). Thickening of the smooth muscle layer of the ISV was found in patients with varicocele (fig. 2, A) compared to the ISV muscle layer of the control group (fig. 2, B). Moreover, expression of HIF-1 $\alpha$  in

TABLE 1. Relative HIF-1 $\alpha$  expression

Case No.	Control Group	Study Group
1	10.07	78.36
2	14.44	69.98
3	7.23	78.02
4	13.76	83.18
5	10.00	75.77
6	10.01	70.76
7	—	53.67
8	—	75.48
Mean	10.92	73.15
SD	2.70	8.93

Values of HIF-1 $\alpha$  expression are normalized relative to  $\alpha$ -tubulin expression,  $p < 0.01$ .

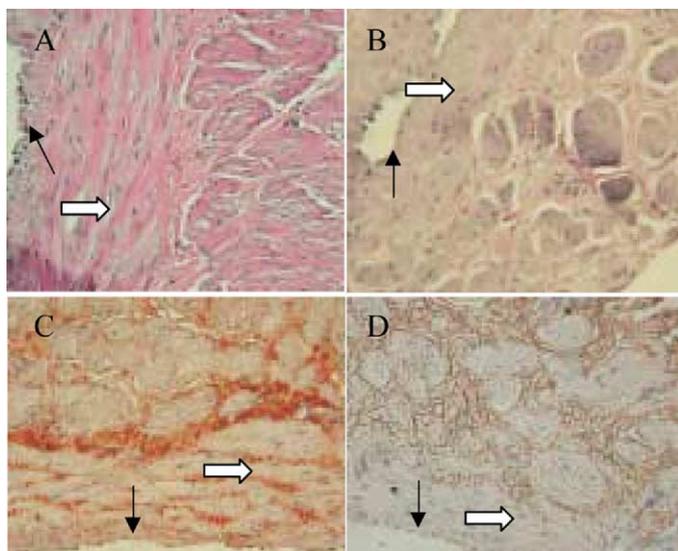


FIG. 2. Micrographs of representative ISV sections after H & E staining or HIF-1 $\alpha$  immunostaining. *A* and *C*, study group (patients with varicocele). *B* and *D*, control group (patients with inguinal hernia). Smooth muscle layer of ISV (white arrows) is thickening in study group compared to control group. Intensity of HIF-1 $\alpha$  immunostaining (brown deposition) localized in smooth muscle is markedly higher in study group (*C*) than in control group (*D*). Black arrows indicate endothelium of ISV. Reduced from  $\times 200$ .

the smooth muscle of the ISV was higher in patients with varicocele (fig. 2, *C*) than in the control group (fig. 2, *D*).

## DISCUSSION

Varicocele is the most common etiological factor associated with male infertility.<sup>1–6</sup> Despite decades of studies, it is still unclear how varicocele develops, whether varicocele actually causes testicular dysfunction and, if so, how varicocele causes that dysfunction. Several theories have been postulated to explain how varicocele may lead to infertility. Varicocele has been thought to induce the reflux of adrenal or renal metabolites, an increase in testicular temperature,<sup>3,5</sup> venous stasis leading to tissue hypoxia,<sup>2,3,9</sup> impairment of the hypothalamus-gonadal axis, a negative impact of reactive oxygen species on testicular function<sup>4,7</sup> and germ cell apoptosis.<sup>6,8</sup>

Hypoxia initiates a cascade of molecular pathways, including those involved in angiogenesis, glycolysis and changes in microenvironmental pH.<sup>9–14</sup> Researchers have hypothesized that poor venous return, which increases the volume of blood in the testis and results in venous stasis, leads to a decrease in testicular oxygen levels. In animal studies hyperbaric oxygenation can effectively alleviate and even eliminate chronic ischemia, anoxia and microcirculatory dysfunction in testicular tissues with varicocele and, therefore, can protect the structure and function of testes.<sup>2</sup>

HIF-1 binds to the VEGF gene and is a key transcriptional mediator of VEGF mediated angiogenesis in response to hypoxia and other metabolic adaptations.<sup>9–11</sup> HIF-1 $\alpha$  is a basic helix-loop-helix transcription factor composed of 2 subunits, HIF-1 $\alpha$  and HIF-1 $\beta$ .<sup>10–14</sup> The activity of HIF-1 is mainly determined by HIF-1 $\alpha$ , whose transcriptional activity and DNA binding ability are strictly regulated by oxygen concentration and other stimuli such as metal ion, nitric oxide, reactive oxygen species and many growth factors.<sup>12–16</sup>

HIF-1 $\alpha$  aids cells in adapting to hypoxia by enhancing cell survival and proliferation.<sup>9–16</sup> The relationship between HIF-1 $\alpha$  induction, VEGF expression and angiogenesis has been previously studied in experimental varicocele of the testis in rats.<sup>9</sup>

In the present study we demonstrate that HIF-1 $\alpha$  is expressed at a higher level in the ISV of patients with varicocele (compared to control patients) and that HIF-1 $\alpha$  expression is predominantly located in the smooth muscle layer of the ISV. These findings directly show that hypoxia occurs in the ISV of patients with varicocele and indirectly suggest hypoxia of testicular tissues in these patients. Thus, hypoxia may be a major factor causing hypospermatogenesis in patients with varicocele, causing male infertility. To our knowledge this is the first report describing the expression of HIF-1 $\alpha$  in the ISV of patients with varicocele. These findings may open new avenues to prevent the formation or recurrence of varicocele by inhibiting HIF-1 $\alpha$  expression and/or reducing hypoxia in the testes.

## CONCLUSIONS

This study shows a high HIF-1 $\alpha$  expression in the ISV of patients with varicocele. This demonstrates that hypoxia related pathophysiological changes have occurred to adapt to hypoxic stress in the ISV of patients with varicocele, and suggests the occurrence of hypoxia in the testicular tissue. Thus, it would be of interest to investigate whether decreasing HIF-1 $\alpha$  activation and testis hypoxia could reduce the recurrence of varicocele. Additional studies are also needed to clarify the relationship between testis hypoxia and male infertility in patients with varicocele.

### Abbreviations and Acronyms

AP	=	alkaline phosphatase
HIF-1 $\alpha$	=	hypoxia-inducible factor-1 $\alpha$
ISV	=	internal spermatic vein
VEGF	=	vascular endothelial growth factor

## REFERENCES

- Redmon, J. B., Carey, P. and Pryor, J. L.: Varicocele—the most common cause of male factor infertility? *Hum Reprod Update*, **8**: 53, 2002
- Guo, Y. L., Zhou, S. W. and Zhang, X. P.: Effects of hyperbaric oxygenation on rabbits' testes with varicocele. *Zhonghua Nan Ke Xue*, **9**: 443, 2003
- Naughton, C. K., Nangia, A. K. and Agarwal, A.: Pathophysiology of varicoceles in male infertility. *Hum Reprod Update*, **7**: 473, 2001
- Nagler, H. M.: Varicocele. Where, why and, if so, how? *J Urol*, **172**: 1239, 2004
- Schoor, R. A., Elhanbly, S. M. and Niederberger, C.: The pathophysiology of varicocele-associated male infertility. *Curr Urol Rep*, **2**: 432, 2001
- Marmar, J. and Benoff, S.: Editorial: New scientific information related to varicoceles. *J Urol*, **170**: 2371, 2003
- Mostafa, T., Anis, T. H., El-Nashar, A., Imam, H. and Othman, I. A.: Varicolectomy reduces reactive oxygen species levels and increases antioxidant activity of seminal plasma from infertile men with varicocele. *Int J Androl*, **24**: 261, 2001
- Tanaka, H., Fujisawa, M., Tanaka, H., Okada, H. and Kamidono, S.: Apoptosis-related proteins in the testes of infertile men with varicocele. *BJU Int*, **89**: 905, 2002

9. Kilinç, F., Kayaselcuk, F., Aygun, C., Guvel, S., Egilmez, T. and Ozkardes, H.: Experimental varicocele induces hypoxia inducible factor-1 $\alpha$ , vascular endothelial growth factor expression and angiogenesis in the rat testis. *J Urol*, **172**: 1188, 2004
10. Zhong, H., Semenza, G. L., Simons, J. W. and De Marzo, A. M.: Up-regulation of hypoxia-inducible factor 1 alpha is an early event in prostate carcinogenesis. *Cancer Detect Prev*, **28**: 88, 2004
11. Yeo, E. J., Chun, Y. and Park, J. W.: New anticancer strategies targeting HIF-1. *Biochem Pharmacol*, **68**: 1061, 2004
12. Nordal, R. A., Nagy, A., Pintilie, M. and Wong, C. S.: Hypoxia and hypoxia-inducible factor-1 target genes in central nervous system radiation injury: a role for vascular endothelial growth factor. *Clin Cancer Res*, **10**: 3342, 2004
13. Pugh, C. W. and Ratcliffe, P. J.: Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med*, **9**: 677, 2003
14. Horiuchi, A., Imai, T., Shimizu, M., Oka, K., Wang, C., Nikaido, T. et al: Hypoxia-induced changes in the expression of VEGF, HIF-1 alpha and cell cycle-related molecules in ovarian cancer cells. *Anticancer Res*, **22**: 2697, 2002
15. Zhong, H., De Marzo, A. M., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D. et al: Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res*, **59**: 5830, 1999
16. Zhong, H., Agani, F., Baccala, A. A., Laughner, E., Rioseco-Camacho, N., Isaacs, W. B. et al: Increased expression of hypoxia inducible factor-1alpha in rat and human prostate cancer. *Cancer Res*, **58**: 5280, 1998
17. Liguori, G., Trombetta, C., Garaffa, G., Bucci, S., Gattuccio, I., Salame, L. et al: Color Doppler ultrasound investigation of varicocele. *World J Urol*, **22**: 378, 2004
18. Tasci, A. I., Resim, S., Caskurlu, T., Dincel, C., Bayraktar, Z. and Gurbuz, G.: Color doppler ultrasonography and spectral analysis of venous flow in diagnosis of varicocele. *Eur Urol*, **39**: 316, 2001
19. Ozgocmen, S., Kocakoc, E., Kiris, A., Ardicoglu, A. and Ardicoglu, O.: Incidence of varicoceles in patients with ankylosing spondylitis evaluated by physical examination and color duplex sonography. *Urology*, **59**: 919, 2002
20. Hopps, C. V., Lemer, M. L., Schlegel, P. N. and Goldstein, M.: Intraoperative varicocele anatomy: a microscopic study of the inguinal versus subinguinal approach. *J Urol*, **170**: 2366, 2003

## EDITORIAL COMMENT

Lee et al describe an increase in HIF-1 expression in smooth muscle cells of the ISV in men with varicocele. They suggest that decreasing HIF-1 activation could reduce the occurrence of varicocele. This is an interesting hypothesis, however another more plausible hypothesis is that activation of HIF-1 is beneficial to varicocele. Chronic hypoxia activates HIF-1, resulting in an increase in the expression of vascular endothelial growth factor (stimulates angiogenesis), erythropoietin (stimulates erythropoiesis), glycolytic enzymes and glucose transporters. Thus, activation of HIF-1 is a compensatory mechanism for hypoxia. The observation that HIF-1 is increased in men with varicocele is not surprising and indicates that cells of the ISV are adapting to a low oxygen environment. Decreasing HIF-1 may only make matters worse. Lee et al further suggest that infertility associated with varicocele may be due to hypoxia of the testis. Indeed, the testis has been described as functioning on the brink of hypoxia and changes in oxygen levels may adversely affect spermatogenesis. It is interesting to note that in the normal murine testis HIF-1 has been described in spermatocytes<sup>1</sup> and Leydig cells,<sup>2</sup> suggesting a role in testicular homeostasis.

**Jeffrey J. Lysiak**  
Department of Urology  
University of Virginia  
Charlottesville, Virginia

1. Marti, H. H., Katschinski, D. M., Wagner, K. F., Schaffer, L., Stier, B. and Wenger, R. H.: Isoform-specific expression of hypoxia-inducible factor-1alpha during the late stages of mouse spermiogenesis. *Mol Endocrinol*, **16**: 234, 2002
2. Turner, T. T., Zheng, S., Palmer, L. A. and Lysiak, J. J.: Hypoxia inducible factor-1 $\alpha$  expression in the murine testis. Presented at the 37th Annual Meeting of the Society for the Study of Reproduction, *Biol Reprod*, special issue: 95, abstract 13, 2004