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Expression of the proto-oncogene *c-fos* and the immunolocalization of c-fos, phosphorylated c-fos and estrogen receptor beta in the human testis

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Summary. Spermatogenesis is under the control of a complex endocrine and paracrine system, including estrogen receptor (ER) signaling. In many target cells, ER promotes the transcription of *c-fos* and other proto-oncogenes to regulate cell growth and differentiation. Thus, in this study we evaluated the expression of the proto-oncogene *c-fos* and the immunolocalization of c-fos, phosphorylated c-fos and ERbeta proteins in the human testis. Testis tissue samples were obtained from 12 men undergoing orchiectomy as adjuvant treatment for prostate cancer, and were stained by immunohistochemistry for c-fos, phosphorylated c-fos and ERbeta localization. Both forms of c-fos proteins were immunoreactive, mainly in germ cells (spermatogonia, spermatocytes and spermatids) and Sertoli cells, while ERbeta was primarily present in somatic cells (Leydig, Sertoli and myofibrillar cells). In addition, testicular biopsies obtained from infertile men with obstructive azoospermia/normal spermatogenesis (n=8) or non-obstructive azoospermia/severely impaired spermatogenesis (n=12) were evaluated for c-fos and ERbeta mRNA levels using real time polymerase chain reaction. The expression of c-fos mRNA was significantly lower (fold change = 0.08, $p<0.05$) whereas that of ERbeta mRNA was higher (fold change = 9.43, $p<0.05$) in the testis of men with non-obstructive azoospermia compared to those with obstructive azoospermia. These findings suggest a complex interrelation between estrogen signaling and *c-fos* transcriptional activity within the human testis, with the increase of ERbeta mRNA being putatively a compensatory mechanism for lower c-fos expression in infertile men with damaged spermatogenesis.

Key words: Proto-oncogene c-fos, ERbeta, Testis, Fertility

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Introduction

The testes are glands with endocrine and exocrine functions, the latter being characterized by sperm formation, or spermatogenesis. This is under the control of a complex system involving endocrine and paracrine factors produced in the hypothalamus-pituitary-testis axis that act direct or indirectly on the testis (McLachlan et al., 1996; Huleihel and Lunenfeld, 2004).

Testosterone and its metabolites (dihydrotestosterone and estradiol) have effects on all steps of male reproductive function (Holdcraft and Braun, 2004). Testosterone and dihydrotestosterone, via androgens receptor (AR), are involved in the process of sexual differentiation and maturation, development of secondary sexual characteristics and promotion and maintenance of normal spermatogenesis. Estrogens derived from androgens also participate in the embryonic testis development, activity and growth of adult prostate, and finally, regulation of gonadotropins and gonadotropin releasing hormone (GnRH) secretion (Griffin and Ojeda, 2004).

Estrogen receptors (ER) alpha and beta are present in the testis, efferent ductules and epididymis of many species (Hess and Carnes, 2004). ERalpha is absent in human germ cells and Sertoli cells (Saunders et al., 2002) and present in the efferent ductules, where it regulates the expression of proteins involved in fluid resorption and ion transport. However, ERbeta is

expressed in all interstitial cells and germ cells with the exception of elongated spermatids (Makinen et al., 2001; Saunders et al., 2002; Gaskell et al., 2003; Aschim et al., 2004; Shapiro et al., 2005). The function of ERbeta is still unknown because ERbeta knockout mice are fertile and seem to have normal testis and epididymis (Krege et al., 1998).

While gonadotropins and testosterone have well-defined roles in the control of spermatogenesis, knowledge is limited when referring to estrogens and intrinsic cell factors. Of importance, estrogen effects are partly mediated by proto-oncogenes (Matthews et al., 2006), which are normal cellular genes homologous to viral oncogenes. The products of proto-oncogenes are important regulators of biological processes and appear to be involved in the events that serve to maintain cell differentiation (Muller and Wagner, 1984) and proliferation (Holt et al., 1986).

The nuclear proto-oncogene *c-fos* was found to be necessary for spermatogenesis by studies of mice homozygous for a null mutation at that locus (Johnson et al., 1992). In mammalian testes, some proto-oncogenes, including c-fos, present stage specific activity during the process of germ cell differentiation (Kierszenbaum, 1994). There is *c-fos* activity in testicular parenchyma (Hall et al., 1991), germline compartments (Norton and Skinner, 1992) and particularly pre-meiotic cells (Kierszenbaum, 1994).

Investigations of proto-oncogenes in relation with testicular activity and possible relationships with steroid hormones have been restricted to rats and mice (Schuchard et al., 1993; Subramaniam et al., 1993) or studies *in vitro*, which probably differ from a physiologic environment (Cobellis et al., 1997). Therefore, an *in vivo* investigation of *c-fos* expression and the presence and distribution of c-fos and ERbeta proteins in the various cell types of testicular parenchyma could contribute to a better understanding of human testicular physiology.

Thus, the aim of the present study was to evaluate the immunolocalization of c-fos, phosphorylated c-fos and ERbeta proteins in the human testis. In addition, the relative transcription levels of *c-fos* and *ERbeta* genes were investigated in testicular samples of infertile men with preserved vs. disrupted spermatogenesis.

Materials and methods

Patients and tissue samples

Approval by the local Human Investigation Committee was granted and all participants gave their written informed consent to participate in the study.

The study included 12 men with previously proven fertility and normal spermatogenesis, aged 64 to 91 years (median 74 years) submitted to orchiectomy for adjuvant treatment of prostate cancer (Group I). Orchiectomy was performed under regional anesthesia, by subcapsular approach, with complete removal of the testicular parenchyma. All samples were evaluated by routine histology, which confirmed normal testicular architecture and full spermatogenesis.

The study also included 20 azoospermic men (age range 23 to 38 years, median 30.5 years) submitted to testicular biopsy under local anesthesia, as part of infertility diagnostic work-up (Group II). The patients were subdivided into two groups according to the presence of obstructive azoospermia (i.e., with normal spermatogenesis, n=8) or non-obstructive azoospermia (with severely impaired spermatogenesis, ranging from maturation arrest to Sertoli cell only; n=12). The histological diagnosis in all cases was reviewed and confirmed independently by two experienced pathologists.

One portion of each tissue sample was snap frozen in liquid nitrogen and stored at -80°C until total RNA extraction and mRNA analysis. A second portion of each sample was fixed in Bouin's solution for 15 min and transferred to 70% ethanol, followed by dehydration in 70% to 99% ethanol and xylene and paraffin inclusion, for subsequent histological diagnosis and/or immunohistochemistry. Only samples from whole testis were available for immunohistochemistry because testicular biopsy specimens were very limited in size and additional fragments could not be obtained due to ethical constrains.

Immunohistochemistry

Bouin fixed, paraffin-embeddes specimens were cut into 4 µm slices, which were stained by immunohistochemistry using the avidin-biotin-peroxidase method. All samples and controls were processed together. Non-specific staining was avoided by exposure to 0.6% H_2O_2 in methanol to block endogenous peroxidase and antigen recovery was enhanced by 20 min microwave boiling, followed by 30 min incubation with normal goat serum. For the localization of c-fos, a polyclonal antibody made in rabbit (Calbiochem, Darmstadt, Germany) was diluted 1:80 and applied during 48 hours at 4°C. This antibody recognizes c-fos protein regardless of its phosphorylation state. For phosphorylated c-fos localization, a polyclonal antibody anti-human protein c-fos, phosphorylated at threonine in position 272, made in rabbit (Abcam, Cambridge, UK) was diluted 1:80 and applied during 48 hours at 4°C. Finally, for ERbeta localization, a polyclonal antibody anti-human protein ERbeta made in mouse (Novocastra Laboratories, Newcastle, UK) was diluted 1:25 and applied during 48 hours at 4°C.

Sections were treated with biotinylated goat anti-rabbit IgG (1:50, for c-fos and phosphorylated c-fos) or goat anti-mouse IgG (1:50, for ERbeta) for 60 min at room temperature and incubated with the avidin-biotin-peroxidase complex (Vectastain® ABC Kit, Vector, Burlingame, CA, USA) for 60 min. Peroxidase activity was visualized by exposing the slices for an optimal time

to 1 mg/ml 3,3'-diaminobenzidine tetra-hydrochloride (Sigma Chemical Co., St. Louis, MO) in PBS containing 0.01% H_2O_2. The sections were then counterstained with haematoxylin, dehydrated and mounted. For negative controls, non-immune serum was used in place of the primary antibody.

Immunostaining was documented under light microscopy with 400x magnification by two independent observers. Tubular and interstitial compartments were evaluated separately according to Clermont (1963) and the intensity of immunostaining was scored as always negative (-), mostly negative (+/--), mostly positive (++/-) or always positive (+++).

Isolation of RNA and synthesis of complementary DNA

Total RNA was isolated from the frozen samples after homogenization with the Trizol® reagent (Invitrogen, São Paulo, Brazil). The homogenate was incubated with chloroform, shaken vigorously by hand for 15 seconds and centrifuged at no more than 12,000xg for 15 minutes at 4°C. The aqueous phase which contains the RNA was transferred to a fresh tube and mixed with isopropyl alcohol for precipitation. Another centrifuge formed a gel-like pellet on the bottom of the tube. The supernatant was removed and the RNA pellet was washed once with 75% ethanol. At the end, the RNA pellet was briefly dried, redissolved in deionized water, quantified using a spectrophotometer (GeneQuant®, Pharmacia Biotech, Uppsala, Sweden) and stored at -80°C.

First strand complementary DNA (cDNA) was synthesized from 2 µg total RNA using the Superscript first-strand synthesis system purchased from Invitrogen (Carlsbad, CA, USA). After denaturing the template RNA and primers at 70°C for 10 min, 50 U reverse transcriptase was added in the presence of RT buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 2.5 mM $MgCl_2$, 0.5 mM dNTP mix and 40 U RNAse inhibitor. The mixture (20 µl) was incubated at 42°C for 55 min, then heated at 70ºC to stop the reaction and stored at -20°C.

Real time PCR

Real time polymerase chain reaction (Real Time PCR) was carried out in an Abi-Prism 7700 Sequence Detection System using the fluorescent dye SYBR Green Master Mix (Applied Biosystems, Foster City, CA). All samples were run in duplicate on 96-well optical PCR plates (Applied Biosystems) in a final reaction volume of 25 µl. The PCR parameters were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min.

The primers used for PCR amplification of *c-fos* and ERbeta are listed in Table 1. The gene encoding the ribosomal protein S26 was used as an internal control. Primers were designed to span intron-exon borders and thus anneal only to cDNA. No amplification of fragments occurred in negative control samples prepared without reverse transcriptase. The specificity of PCR products was confirmed by the single peak dissociation curves and by acrylamide gel electrophoresis showing that the amplicons had the predicted molecular weight. The relative gene expression was calculated by use of the $2^{-\Delta\Delta CT}$ method, where CT is threshold cycle (Livak and Schmittgen, 2001).

Statistical analysis

The PCR results were analyzed based on the ΔCT, which is the primary source of data variability (Yuan et al., 2006). The CT values were normally distributed and therefore they were summarized as mean ± standard error and differences between the two patient groups were analyzed with the unpaired Student's t test, considering $p<0.05$ as statistically significant (Yuan et al., 2006).

Results

Immunolocalization of c-fos proteins and ERbeta in human testis (Group I)

Nuclear immunoreactivity for c-fos occurred mainly in type B spermatids and Sertoli cells. Sertoli cell intensity varied from intense to moderate and sometimes negative. Spermatogonia, spermatocytes and type A spermatids were mostly negative. When positive, spermatogonia presented nuclei intensely positive while spermatocytes and type A spermatids were weaker. Type C and D spermatids were positive only in their developing flagella. Leydig cells presented nonspecific immunostaining confirmed by negative control and myoid cells were always negative (Fig. 1, Table 2).

As shown in Figure 2, with phosphorylation of the threonine residue at position 232 of c-fos protein, differences in immunoreactivity were notable when compared with total c-fos protein. Types A, B and C spermatids presented their nuclei mostly positive and especially their acrosomes. Type D spermatids were always negative and sometimes it was possible to

Table 1. Oligonucleotides used in Real Time PCR.

	Sequence (5' to 3')	Amplicon size	GenBank Accession No.
c-fos			
forward	tacactccaagcggagacag	84 bp	V01512
reverse	tccttctccttcagcaggtt		
ERbeta			
forward	aaggttagtgggaaccgttg	129 bp	NM001437
reverse	acatccttcacacgaccaga		
S26			
forward	tgtgcttcccaagctgtatgtgaag	75 bp	NM001029
reverse	cgattcctgactactttgctgtgaa		

distinguish when type D spermatids were becoming negative. Sertoli cells were mostly negative to phosphorylated c-fos. All other cell types presented no kind of specific immunostaining (Fig. 2, Table 2).

As for the localization of ERbeta, myoid and Sertoli cells were always positive, but in some seminiferous tubules Sertoli cells were feebly positive. Some spermatocytes and type A spermatids also presented mild nuclear immunoreactivity, but mostly negative. Most Leydig cells presented mildly positive and some strongly positive. All other cell types presented no kind of specific immunostaining (Fig. 3, Table 2).

C-fos and ERbeta gene expression in testicular biopsy samples (Group II)

Figure 4 shows the relative expression of *c-fos* and ERbeta mRNA in testicular biopsies of infertile men

Table 2. Summary of immunolocalization of c-fos, phosphorylated c-fos and ERbeta proteins in the human testis.

Immunolocalization	c-fos	Phosphorylated c-fos	ERbeta
Leydig cell	-	-	+++
Myoid cell	-	-	+++
Sertoli cell	++/-	+/--	+++
Spermatogonia	+/--	-	-
Spermatocytes	+/--	-	+/--
Type A spermatids	+/--	++/-	+/--
Type B spermatids	+++	++/-	-
Type C spermatids	-	++/-	-
Type D spermatids	-	-	-

(-) always negative; (+/--) mostly negative; (++/-) mostly positive; (+++) always positive.

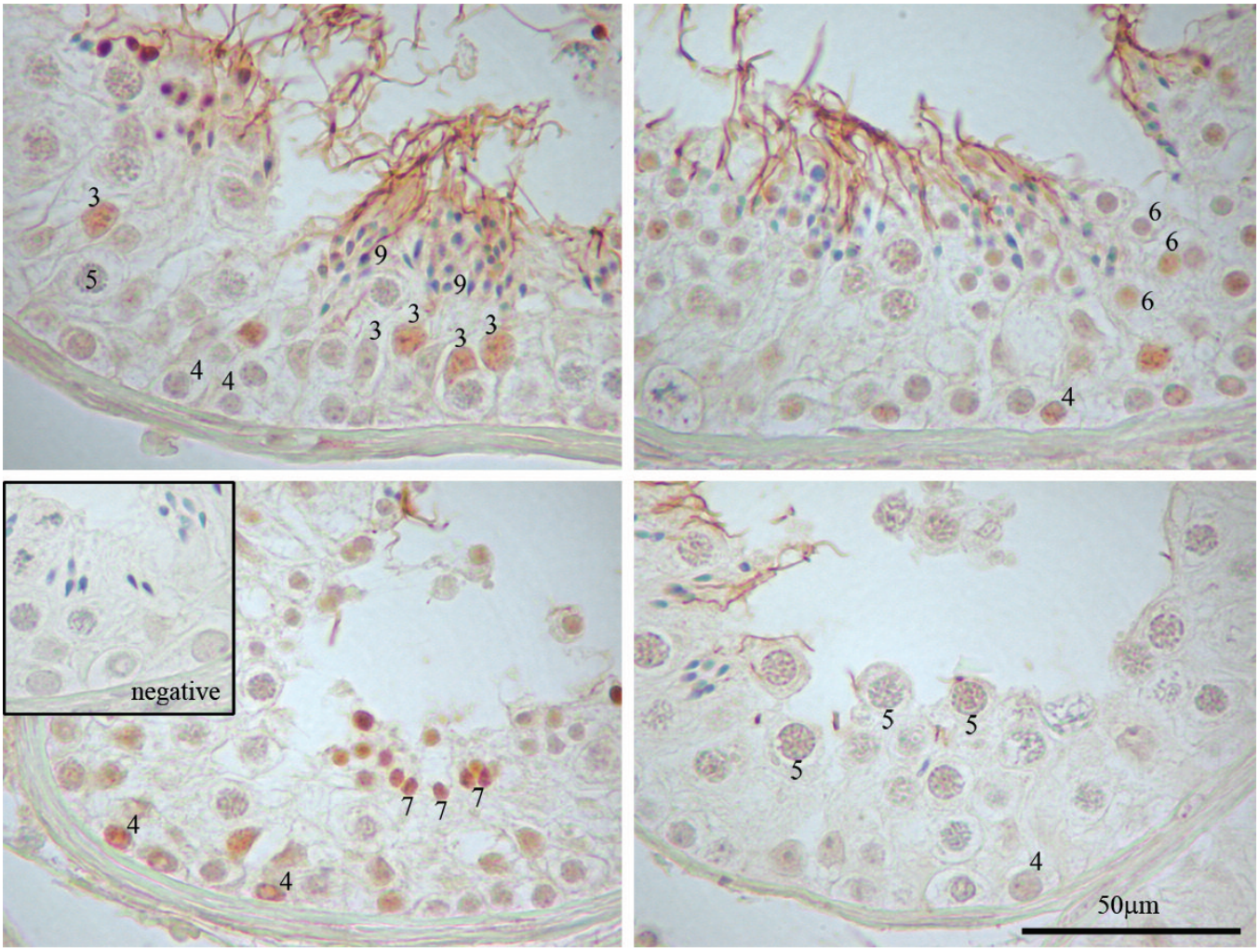

Fig. 1. Immunolocalization of the c-fos protein in the parenchyma of the human testis from orchiectomized men (n=12). The brownish staining indicates occurrence of the c-fos protein. 3: Sertoli cell; 4: spermatogonia; 5: spermatocyte; 6: Type A spermatid; 7: Type B spermatid; 9: Type D spermatid.

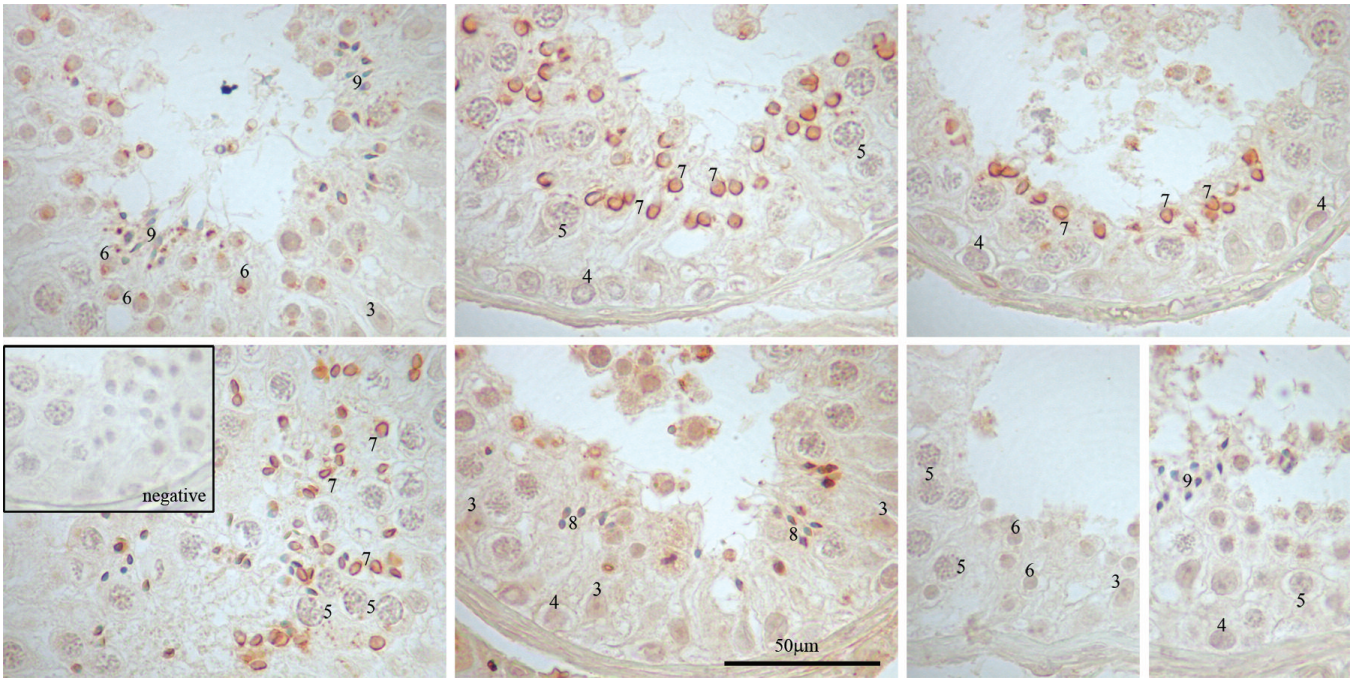

Fig. 2. Immunolocalization of the phosphorylated c-fos protein in the parenchyma of the human testis from orchiectomized men (n=12). The brownish staining indicates occurrence of the phosphorylated c-fos protein. 3: Sertoli cell; 4: spermatogonia; 5: spermatocyte; 6: Type A spermatid; 7: Type B spermatid; 8: Type C spermatid; 9: Type D spermatid.

with obstructive azoospermia/full spermatogenesis vs. non-obstructive azoospermia/impaired spermatogenesis. The expression of *c-fos* mRNA was significantly lower in the testis of men with non-obstructive azoospermia ($1/\Delta CT = 0.15\pm0.11$) than in the testis of men with obstructive azoospermia ($1/\Delta CT = 1.76\pm0.97$, fold change = 0.08, $p<0.05$, Fig. 4A). On the other hand, the relative expression of ERbeta mRNA was significantly increased in the group with non-obstructive azoospermia ($1/\Delta CT = 0.81\pm0.51$) compared to that with obstructive azoospermia ($1/\Delta CT = 0.12\pm0.09$, fold change = 9.43, $p<0.05$, Fig. 4B).

Discussion

The present study firstly shows that the human testis expresses the proto-oncogene *c-fos* and the phosphorylated c-fos protein. Moreover, we have shown that the expression of c-fos is decreased in the testis of infertile men with impaired spermatogenesis compared to those with normal spermatogenesis.

As the mechanisms involved in the control of cell growth and development are deciphered, it becomes obvious that proto-oncogenes exert fundamental roles in the modulation of proliferation and differentiation of normal cells (Schuchard et al., 1993). The proto-oncogene *c-fos* is expressed in the testicular parenchyma of some vertebrate species, such as mouse (Wolfes et al., 1989; Hall et al., 1991; Schultz et al., 1995; Cobellis et al., 1997)), pig (Wolfes et al., 1989; Hall et al., 1991; Schultz et al., 1995; Cobellis et al., 1997), rat (Wolfes et al., 1989; Hall et al., 1991; Schultz et al., 1995; Cobellis et al., 1997) and frog (Wolfes et al., 1989; Hall et al., 1991; Schultz et al., 1995; Cobellis et al., 1997). Schultz et al. (1995) showed how *c-fos* gene expression and protein immunoreactivity changed along successive stages of rat spermatogenesis and that protein expression was not always related to gene transcription. It is known that some mRNA can be produced at the beginning of the spermatogenic cycle and translated at late stages of spermiogenesis (Geremia et al., 1978).

In human seminiferous tubules cultured in vitro (Suomalainen et al., 2004), some spermatogonia and early meiotic spermatocytes expressed cytoplasmic immunostaining for c-fos, whereas Sertoli cells were previously negative and aquired intense nuclear c-fos staining during culture. Interestingly, in the present study we observed that spermatogonia and spermatocytes presented intermittent immunostaining, which may also suggest a stage-specific activation of c-fos in these cells. In addition, we found that type A and type B spermatids have positive nuclear immunostaining for c-fos, thus indicating gene activity in haploid cells. In fact, it has been described that, during spermatogenesis, haploid cells are capable of synthesizing mRNA and proteins (Geremia et al., 1978; Dadoune, 1994).

This is, to the best of our knowledge, the first demonstration of phosphorylated c-fos protein in the

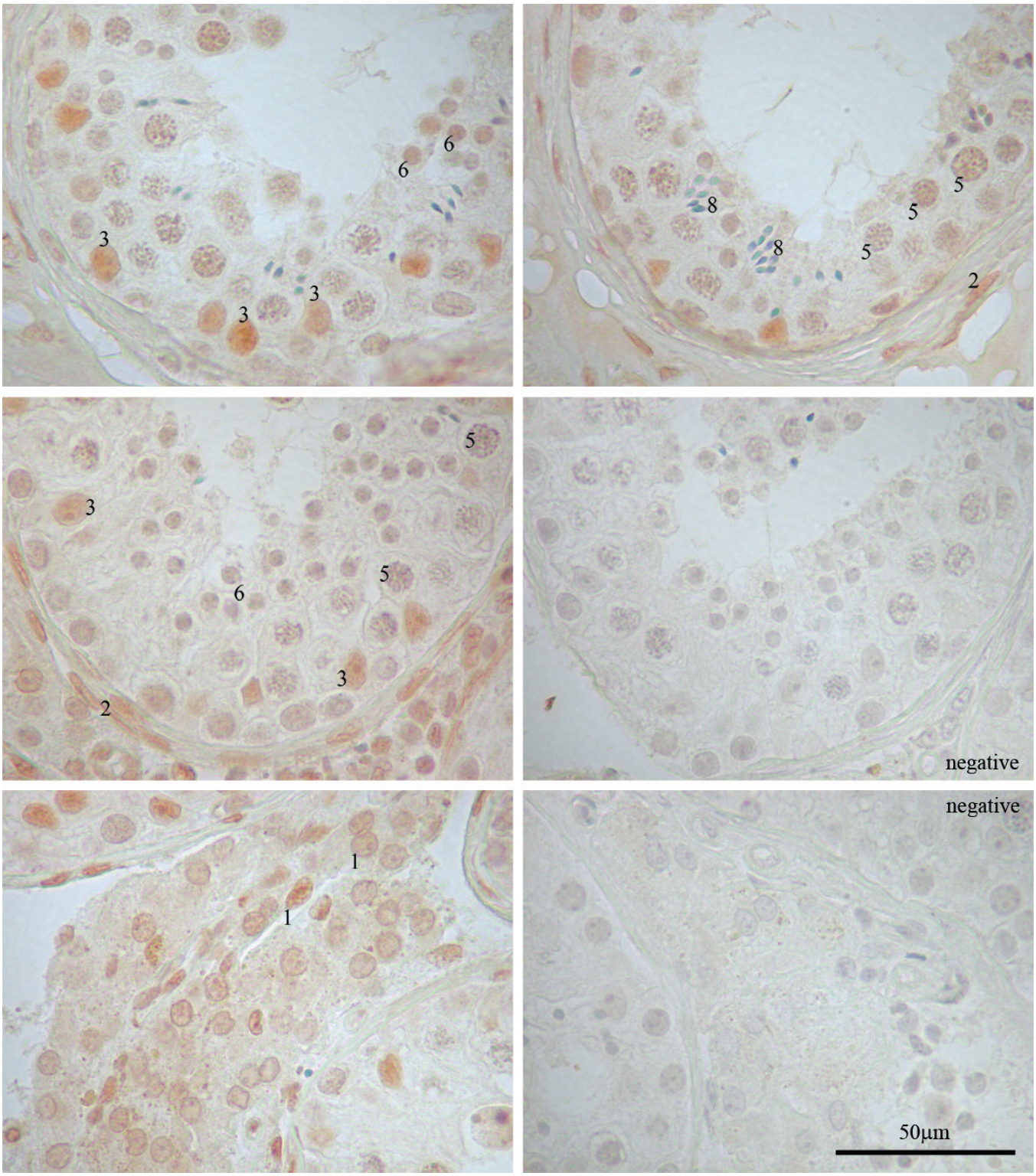

Fig. 3. Immunolocalization of the ERbeta protein in the parenchyma of the human testis from orchiectomized men (n=12). The brownish staining indicates occurrence of ERbeta protein. 1: Leydig cell; 2: myoid cell; 3: Sertoli cell; 5: spermatocyte; 6: Type A spermatid; 8: Type C spermatid.

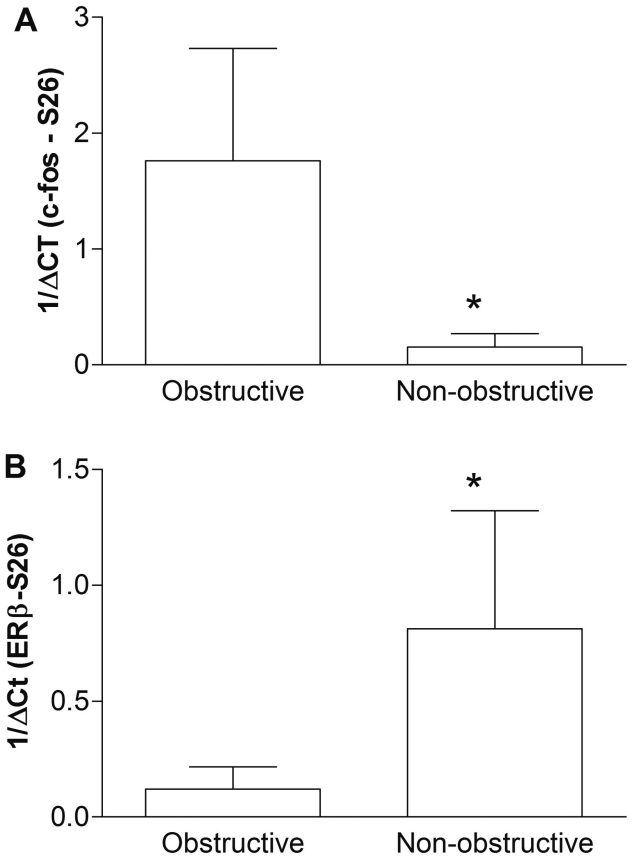

Fig. 4. Expression of c-fos **(A)** and ERbeta **(B)** mRNA in testicular biopsies of infertile men with obstructive (n=8) and non-obstructive (n=12) azoospermia. For better clarity, data are plotted as mean ± standard error of 1/ΔCT, which is directly proportional to the relative gene expression (fold change). *p<0.05 (unpaired t test).

human testis. Kousteni et al. (2003) described that, by estrogen-ER interaction, activation of protein kinases with activity over serine/threonine residues can occur, which would be responsible by c-fos phosphorylation. This mechanism is independent from the classic genomic actions of steroid receptors. Also, phosphorylation of c-fos protein in its transactivation domain augments its transcriptional activity (Monje et al., 2003). As shown here, with phosphorylation of c-fos protein, its staining changed from spermatogonia, spermatocytes and types A and B spermatids to types A, B and C spermatids, which could represent a modulation in its activity by autocrine/paracrine factors. In addition, an intense acrosome immunostaining in types A, B and C spermatids was found. Sertoli cells were immunoreactive to total c-fos but not to the phosphorylated protein, which may represent an inhibition of c-fos protein activity in this cell type.

ERbeta expression in human testis has been described before. Saunders et al. (2002) described intense ERbeta nuclear immunostaining in pachytene spermatocytes and round spermatids. Sertoli cells, spermatogonia and pre-leptotene, leptotene, zygotene and diplotene spermatocytes showed a weaker marcation. Aschim et al (2004) found nuclear immunoreactivity in spermatogonia, spermatocytes and young spermatids. In the present study, we found nuclear immunostaining for ERbeta in Leydig, myoid and Sertoli cells, even though Leydig cells were less immunopositive. Spermatocytes and type A spermatids were mostly negative. The differences between these investigations may be due to different protocols and/or antibodies.

Another interesting finding of the present study was that men with non-obstructive azoospermia had lower expression of *c-fos* and higher expression of ERbeta mRNA in the testis compared with men with obstructive azoospermia. While the changes observed in the former group might be merely a consequence of its disrupted testicular architecture, the present data still leaves open the hypothesis that at least some conditions associated to non-obstructive azoospermia induce a specific downregulation of the local expression of *c-fos*, with the increase of ERbeta mRNA being putatively a compensatory mechanism. This hypothesis, however, remains to be tested in an appropriate experimental model.

Even though more studies are needed to confirm that proto-oncogenes and estrogens are capable of modulating the spermatogenic cycle, the presence of the proto-oncogene *c-fos* in somatic cells and mainly germ cells, and ERbeta in somatic cells let us hypothesize two mechanisms for estrogen actions over spermatogenesis. The first would be mediated by Sertoli cells, where estrogens could alter either gene expression or the transcriptional activity of c-fos protein. The other, an indirect mechanism, represents the interrelationship between somatic and germ cells. Somatic cells, under estrogen influence, could modify germ cell functions by paracrine/juxtacrine factors, which would change gene expression or the transcriptional activity of c-fos protein.

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