Urol Res (1998) 26:407–410
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ORIGINAL PAPER

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Assessment of testicular function in experimental varicocele rats by phosphorus-31 magnetic resonance spectroscopy

Received: 21 October 1997 / Accepted: 23 April 1998

Abstract To assess testicular function in experimental varicocele rats, we used ^{31}P magnetic resonance (MR) spectroscopy and compared MR spectroscopic parameters with flow cytometric DNA analysis. In vivo ^{31}P MR spectroscopy and flow cytometric DNA analysis of testes were performed in 10 sham-operated and 9 induced varicocele rats. Although the testicular phosphomonoester (PM)/ATP ratio did not differ between sham-operated and induced varicocele rats, the phosphodiester (PD)/ATP ratio was significantly reduced and the inorganic phosphate (Pi)/ATP ratio was significantly increased in induced varicocele rats. There was no difference between the right and left sides. DNA flow cytometry showed a decrease in the percentage of haploid cells in induced varicocele rats, regardless of the side. This study indicates that in vivo ^{31}P MR spectroscopy provides valuable information for assessment of testicular function.

Key words Varicocele · Nuclear magnetic resonance · Flow cytometry

Introduction

Varicocele is well known to be the most frequent cause of male infertility [7]. The bilateral, deleterious effects of the unilateral lesion on testicular histology is well described [9, 13]. Elevated testicular temperature, reflux of adrenal or renal metabolites, hypoxia secondary to venous stasis, and hormonal imbalance have been proposed as mechanisms through which spermatogenesis is impaired [11, 19]. However, the precise mechanism by which varicocele induces degeneration is not clearly understood. Recently, MR spectroscopy has been used to quantify intracellular metabolic parameters and to study muscle, brain, kidney, testis and heart of animals [2, 3, 6]. In the present study, we used MR spectroscopy to assess in vivo metabolism of rat testes in induced varicocele and compared testicular MR spectroscopic parameters with flow cytometric DNA analysis.

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Materials and methods

Animals

Young adult male rats (300–350 g) of a Sprague-Dawley strain were purchased from Clea Japan (Tokyo, Japan). They were housed 3–5 per cage in hanging wire mesh cages under controlled lighting conditions (14 h light, 10 h dark) and temperature (22 ± 2°C). They were given the commercial diet (CE; Clea Japan) and water ad libitum. The animals were used after a 2-week acclimatization period.

Methods

In the induced varicocele group, 20 animals were anesthetized with sodium pentobarbital. Through an abdominal midline incision, the left renal vein was ligated distal to the left adrenal and testicular veins, to an external diameter of 1 mm. The animals were returned to the vivarium. Eight weeks after the operation, they were anesthetized using sodium pentobarbital. Through a midline incision, the left spermatic vein was observed. Nine animals with dilation of the spermatic vein were included in the induced varicocele group, but the remainder were excluded. In sham operation group, 10 animals were subjected to an operation similar to that of the varicocele group, except that left renal vein was not ligated.

Nine rats in the varicocele group and 10 in the sham operation group were examined with ^{31}P MR spectroscopy at 8 weeks after the first surgery. Spectra were obtained on an Ohtsuka Electrics (Osaka, Japan) BEN 170/200 spectrometer/imager with a 4.7-T superconducting magnet with a 20-cm bore. Intracellular phosphorus metabolites from rat testes were monitored with a two-turn surface coil with a diameter of 20 mm, tuned and matched to 75.5035 MHz. Field homogeneity was optimized by shimming on the water proton signal. Partially saturated ^{31}P spectra with a repetition time of 1 s were obtained from 500 accumulated free

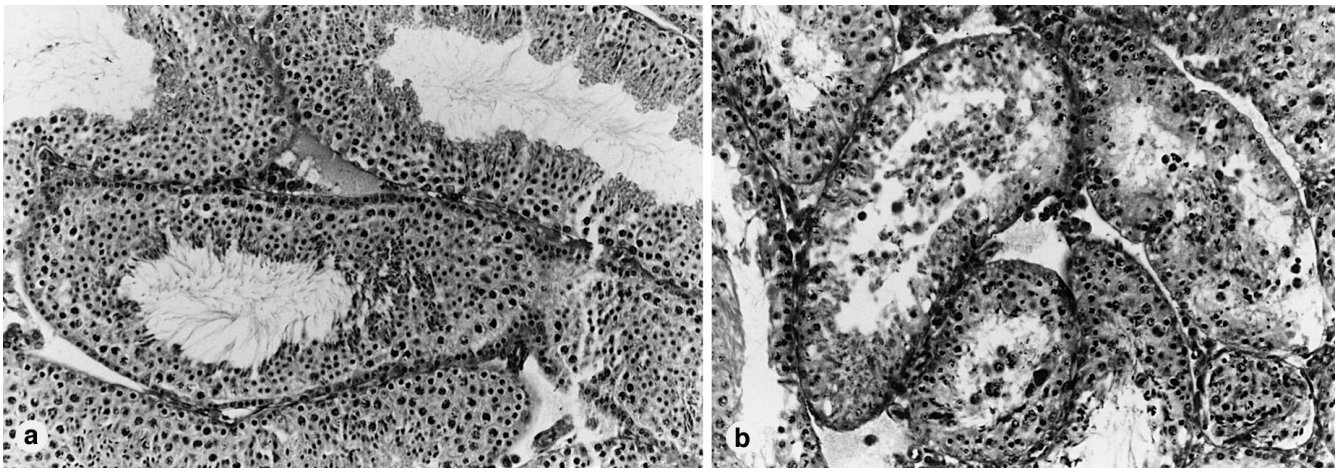

Fig. 1 Testicular histology of rats with sham operation (**a**) and induced varicocele (**b**)

induction decays. In each experiment the surface coil was placed above the testes. The epididymis was positioned relatively far from the surface coil. The spectra were analyzed after exponential multiplication resulting in 10 Hz line broadening. Quantification of the spectra was performed by a cut-and-weigh method [8]. First, a straight baseline as drawn through the noise from both sides of the spectral region of interest. In cases of overlapping peaks the whole area was measured and a second quantification of this area was performed by manual triangulation [18]. The phosphomonoester (PM), inorganic phosphate (Pi), phosphodiester (PD) and three ATP peaks (alpha, beta and gamma) were assigned for analysis. The concentration of the metabolites was expressed relative to the beta phosphate peak of ATP.

After MR spectroscopy, all rats from each group were killed. Bilateral testes were removed, and half of each specimen was fixed in Bouin's solution for routine histologic examination. The rest of each specimen was frozen at −80°C prior to flow cytometric analysis. Before analysis, specimens were quickly thawed in a water bath at 37°C and mechanically minced. Each cell pellet was resuspended in 1 ml nuclear staining solution (50 mg/ml propidium iodide, 1.2% sodium citrate, 0.1% Nonidet P-40) and cells were stained at room temperature for 15 min. Then 250 ml of a solution of ribonuclease B (Sigma, St Louis, Mo.) was added to 250 ml of cell suspension in the nuclear staining solution and the mixture was incubated for an additional 15 min at room temperature. The sample was repeatedly aspirated through a 26-gauge needle and then filtered through 22-μm nylon mesh before analysis. Flow cytometric analysis was performed on a FACScan system (Becton-Dickinson, Sunnyvale, Calif.) with a 488-nm laser beam. A minimum of 20 000 nuclei per sample was measured at a flow rate of no more than 200 events per second for each histogram. The output signals were sorted by a 256-channel analyzer and organized as histograms. Characterization of the cell cycle from the DNA Cell-Cycle Analysis Software (version C) integration programs (Becton-Dickinson). The peak channel number of each cell population was determined and compared with a control sample of known diploid (2C) cells (propidium iodide-stained human lymphocytes). Haploid (1C) and tetraploid (4C) cell populations were defined by peak channel numbers equal to half and twice that of the reference diploid population, respectively. Spermatogonia, secondary spermatocytes, Sertoli cells, Leydig cells and stromal cells are diploid and, by convention, were assigned the 2C channel number. Spermatids and spermatozoa contain half the diploid DNA content, resulting in a 50% decrease in fluorescent intensity and therefore appear in the 1C channel. Primary spermatocytes contain twice the diploid DNA content, resulting in a twofold increase in fluorescent intensity and consequently appear in the 4C channel. The relative percentage of cells corresponding to each ploidy compartment was determined by the same integration programs.

The protocol of the present study was approved by the responsible ethical committee. Furthermore, the Principles of Laboratory Animal Care (NIH publication No. 86–23, 1984) were followed. In addition, all regulations on experimental animal studies recommended by the Japanese law were adopted.

Results

Full spermatogenesis was observed in testes with sham operation (Fig. 1a). In induced varicocele rats, germ cells were exfoliated from seminiferous epithelium and the numbers of spermatozoa and spermatids reduced (Fig. 1b). However, spermatids and spermatozoa were not absent. These changes were observed bilaterally.

Figure 2 shows typical ^{31}P MR spectra for testis in sham-operated and induced varicocele rats. Although the testicular PM/ATP ratio did not differ between sham-operated and induced varicocele groups, the PD/ATP ratio was significantly reduced ($P < 0.05$) and the Pi/ATP ratio was elevated ($P < 0.05$) compared with those of sham-operated group (Table 1). No significant difference was observed in these ratios between the left and right sides (Table 1). As shown in Table 2, the percentage of haploid cells in the varicocele rats was significantly lower than that in the sham-operated rats ($P < 0.05$), whereas no significant difference was found between the left and right testes. The percentage of diploid cells in the induced varicocele rats showed a considerable elevation compared with that in the sham-operated rats ($P < 0.05$). However, there was no significant difference in this percentage between the left and right sides. No significant difference was found between the two groups in the percentage of tetraploid cells.

Discussion

In the ^{31}P MR spectrum of the rat testis, the PM peak mainly represents phosphocholine and phosphoethanolamine [10]. They are precursors in the biosynthesis of phosphatidylcholine (PC) and phosphatidylethanol-

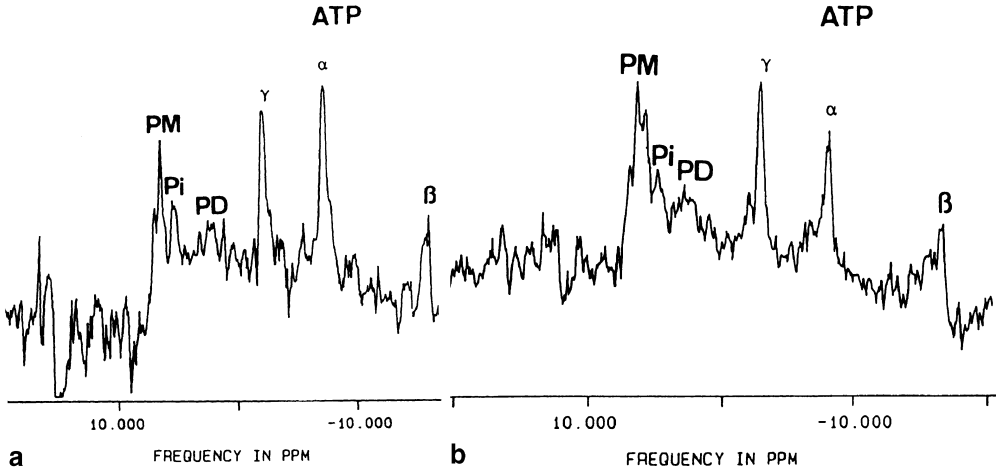

Fig. 2 ^{31}P MR in vivo spectra of testis in sham-operated (**a**) and induced varicocele (**b**) rats

Table 1 Testicular PM/ATP, PD/ATP and Pi/ATP ratios for induced varicocele and sham-operated rats

MR spectroscopic parameters	Induced varicocele (n = 9)		Sham-operated (n = 10)	
	Left	Right	Left	Right
PM/ATP	1.04 ± 0.10	1.03 ± 0.12	1.01 ± 0.12	1.03 ± 0.11
PD/ATP	0.42 ± 0.15[a]	0.41 ± 0.14[a]	0.62 ± 0.18[b]	0.60 ± 0.17[b]
Pi/ATP	0.76 ± 0.13[a]	0.78 ± 0.11[a]	0.59 ± 0.11[b]	0.58 ± 0.12[b]

Each value represents mean ± SD; difference is significant between [a] and [b] ($P < 0.05$) by Student's t-test

Table 2 DNA contents of countered testicular cells in induced varicocele and sham-operated rats

DNA content	Induced varicocele (n = 9)		Sham-operated (n = 10)	
	Left	Right	Left	Right
Haploid cells (1C%)	66 ± 4[a]	68 ± 4[a]	73 ± 5[b]	74 ± 4[b]
Diploid cells (2C%)	20 ± 4[a]	18 ± 5[a]	14 ± 7[b]	12 ± 5[b]
Tetraploid cells (4C%)	12 ± 3	13 ± 2	12 ± 2	12 ± 3

Each value represents mean ± SD; differences are significant between [a] and [b] ($P < 0.05$) by Student's t-test

amine (PE), respectively, following the de novo Kennedy pathway, also known as the cytidine pathway of phospholipid biosynthesis. It is assumed that a high level of phospholipid precursors is related to an increased rate of phospholipid biosynthesis [5, 14]. In experimental cryptorchidism, the transient increase in the PM/ATP ratio is caused by the formation of multinuclear giant cells in the seminiferous tubules [18]. Although the present study showed a reduced percentage of haploid cells in varicocele rats, the formation of multinuclear giant cells was not found. This means that the rate of phospholipid biosynthesis is not high in induced varicocele rats.

The testicular PD peak is mainly represented by glycerophosphocholine (GPC) and of a much smaller amount of glycerophosphoethanolamine (GPE) [15]. These components are both catabolic products of PC and PE, respectively. Glycerophospholipids are broken down by the activity of phospholipase A1, A2 and lysophospholipase. However, considering the large difference in GPC and GPE concentrations in contrast to the small difference in PC and PE [15], it seems that GPC plays another role in testicular metabolism. High concentrations of GPC have been found in seminal fluid [1] and a positive correlation exists between the concentration of GPC in seminal fluid and sperm motility [12]. GPC is probably secreted into the seminal fluid during spermatid development and/or spermiation. Since the PD/ATP ratio is high when spermiation is observed [17], the testicular PD/ATP ratio is considered to be an available marker for the presence of spermatids and/or spermiation. In the present study, the percentage of haploid cells was significantly reduced in induced varicocele rats. Therefore, the PD/ATP ratio resulted in a significant reduction.

Inorganic phosphorus appears as the final breakdown product originating from several phosphorus-containing compunds [4]. In the hypoxic condition, the reduced supply of oxygen to the tissue makes it impossible for the mitochondria to maintain normal ATP levels because of impaired oxidative phosphorylation [16]. Thus, ATP concentrations decrease while the concentration of the waste product of continued metabolic processes, Pi, increases [16]. In induced varicocele rats, the Pi/ATP ratio significantly increased and the percentage of haploid cells significantly decreased. This finding may be indicative of hypoxic changes inside the seminiferous tubules.

Our results indicate that ^{31}P MR spectroscopy provides valuable information for assessment of testicular function in experimental varicocele rats. To what extent the PM/ATP, PD/ATP and Pi/ATP ratios can be used to differentiate the various types of impairment of spermatogenesis in the human testis still needs to be investigated. In patients with a varicocele, ^{31}P MR spectroscopy may be used to monitor improvement of testicular function after the treatment.

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