

## Testicular interleukin-6 response to systemic inflammation

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**ABSTRACT.** Spermatogenesis is a highly controlled process of proliferation, meiosis, and differentiation. Systemic infection and chronic inflammation can impair testicular steroidogenesis and spermatogenesis. In this study, we examined the effect of systemic infection – intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) – on the expression levels of IL-6 in the testis of sexually immature and adult mice. IL-6 levels in testicular homogenates of immature mice were significantly higher than in mature mice (both protein and RNA levels), before and after LPS injection. Injection of LPS (i.p.) into mature mice over 3 hours, significantly increased testicular IL-6 protein and mRNA levels (as demonstrated by ELISA and RT-PCR respectively) compared to the control group. Injection of LPS over 24 hours significantly increased IL-6 mRNA expression, but it did not significantly affect IL-6 protein levels in the homogenates. In contrast, stimulation of immature mice with LPS (2, 20 or 100 µg/mL) over 3 hours or LPS (2 or 20 µg/mL) over 24 hours, significantly increased testicular IL-6 (both protein and mRNA expression). The levels of testicular IL-6 (protein) in the homogenates were not significantly increased after stimulation with 100 µg/mL over 24 hours, but they were significantly increased at the mRNA level. Our results demonstrate, for the first time, the over-expression of IL-6 in testicular homogenates of mature and immature mice following systemic inflammation (i.p. injection of LPS). These results suggest the possibility of the involvement of systemic infection/inflammation, through the elevation of testicular IL-6, in testicular functions, which may affect male fertility. Also, high levels of IL-6 during pathological conditions, could play a role in protecting testicular tissue.

**Keywords:** interleukin-6, Sertoli cells, Leydig cells, germ cells, testis, LPS

Spermatogenesis is a highly controlled process of proliferation, meiosis, and differentiation, which occurs in a number of sets of spermatogenic cell associations or stages [1, 2]. In addition to the regulatory effect of gonadotropins and androgens in the initiation and maintenance of spermatogenesis, a number of cytokines are also involved in the regulation of various differentiation steps in this process [3-5].

Clinical reports indicate that systemic infection and chronic inflammation can impair testicular steroidogenesis and spermatogenesis, leading to temporary or permanent fertility problems [6-10]. Injection of an animal subject with lipopolysaccharide (LPS), an endotoxin present in the outer cell wall of the gram-negative bacteria, resulted in inhibition of gonadotropin secretion by the pituitary, and testosterone secretion by Leydig cells [10, 11]. In addition, LPS induced an acute inflammatory response, mediated by an increased proinflammatory cytokine secretion – mainly tumor necrosis alpha (TNF- $\alpha$ ), interleukin-(IL)-1 and IL-6 – by the immune system [12]. These cytokines could inhibit testicular steroidogenesis directly, or indirectly by

inducing glucocorticosteroid production following activation of the hypothalamic-pituitary-adrenal axis [4, 13, 14]. In the testicular compartments, LPS may induce various cell types of immune and non-immune origin to produce cytokines. In the testicular interstitial compartment, Leydig cells and macrophages are the main responsive cells. In the seminiferous tubules, Sertoli cells, germ cells and differentiated germ cells, and peritubular cells produce cytokines [1, 4, 14]. It was demonstrated that Sertoli cells secrete IL-6, which was increased following stimulation with LPS, latex beads, residual bodies, low levels of testosterone and FSH [15-17]. The levels of IL-6 varied throughout different stages of the seminiferous epithelium cycle; high levels were observed in stages II-VI and the lowest in stages VII-VIII [15]. Leydig cells also express IL-6 following stimulation with LH, LPS and IL-1 *in vitro* [18, 19]. Testicular macrophages were shown to secrete IL-6 and other cytokines following stimulation with LPS under *in vitro* conditions [20].

IL-6 has been suggested to be a potent inhibitor of meiotic DNA synthesis within the seminiferous epithelium [21], and a stimulator of transferrin production by Sertoli cells [18].

It was also shown to inhibit testosterone secretion by Leydig cells [22].

In the present study, we examined the effect of systemic infection (i.p. injection of LPS) on the expression levels of IL-6 in the testis of sexually immature and adult mice.

## MATERIALS AND METHODS

### Materials

Interleukin-6 levels in testicular homogenates were examined using a murine IL-6 Eli-pair kit (Diaclone, Besançon, France). This kit recognizes both natural and recombinant murine IL-6, and has no cross-reactivity with other murine cytokines. The range of the standard curve was 2 to 500 pg/mL, and the sensitivity of the kit was < 32 pg/mL.

### LPS injection

The investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction. Sexually mature (adults) (8-10 weeks old) and sexually immature (2 weeks old) Balb/c mice were used. At the age of 2 weeks, even though Sertoli cells are almost completely differentiated, the spermatogenic process has not yet been completed and mice are not producing spermatozoa [23]. Three mice were examined in each experiment and each experiment was repeated more than 6 times.

Mice were injected (i.p.) with 2, 20 or 100 µg/mL/100 µL of LPS or saline (control). Three hours or 24 hours later, mice were sacrificed by CO<sub>2</sub> asphyxiation, and testicular tissues were immediately separated and stored at -70 °C for RNA extraction or for homogenate preparation.

### Preparation of testicular homogenates

Testicular homogenates were prepared from immature and mature mice. A single testis from each mature and two testes from each immature mouse were prepared and examined separately. The tunica albuginea was removed from the testis and remaining testicular tissue was homogenized in 0.8 mL cold PBS over ice. At the end of the homogenization process, the mixture was centrifuged at 13 000 RPM for 15 min. and the supernatant was collected and stored at -70 °C. Total protein was investigated using Biorad reagent. IL-6 levels were measured using a specific ELISA kit.

### Extraction of total RNA and RT-PCR analysis

Total RNA was extracted from mouse testis using the EZ RNA Reagent protocol (Biological Industries, Beit Haemek, Israel). First-strand complementary DNAs (cDNAs) were synthesized from 2.5 µg total RNA with 0.5 µg random oligonucleotide primers (Roche Molecular Biochemicals, Mannheim, Germany) and 200 U of Moloney-Murine Leukemia Virus-Reverse Transcriptase (M-MLV-RT; Life Technologies, Inc., Paisley, Scotland, UK) in a total volume of 20 µL Tris-HCl-MgCl reaction buffer, supplemented with DTT, dNTPs (0.5 mmol/L; Roche Molecular Biochemicals) and RNase inhibitor (40 U; Roche Molecular Biochemicals). The reverse transcriptase (RT) reaction was performed for 1 h at 37 °C and stopped for 10 min at 75 °C. The volume of 20 µL was subsequently

made up to 60 µL with water. Negative controls for the reverse transcriptase reaction (RT-) were prepared in parallel, using the same reaction preparations with the same samples, without M-MLV-RT.

The PCR, performed subsequently, contained cDNA samples in final dilution of 1:15 with two pairs of oligonucleotide primers (0.9 pmol/µL; 5'AGAGG-GAAATCGTGCGTGAC3'; and 3'GCCGGACTCATCG-TACTCT5' for the mouse β-Actin cDNA sequence, and 5'GACGATACCACTCCCAACAGACC3;' and 3'AT-GCTTAGGCATAACGCACTAGGTT5' for the mouse IL-6 cDNA sequence (Sigma). To assess the absence of genomic DNA contamination in RNA preparations and RT-PCR reactions, PCR was performed with negative controls for the RT reaction (RT-) and without cDNA (cDNA-). The PCR reactions were carried out on a Cyler II System Thermal Cyler (Ericomp, San Diego, CA, USA). Twenty microliters of each PCR product were run on 2% agarose gel, containing ethidium bromide, and photographed under UV light.

The RNA expression was quantified from the different samples of the RT-PCR using TINA software (version 2.10g) (raytest Isotopenmessgeraets, GmbH, Straubenhardt, Germany).

### Evaluation of results

Each experiment included three to five mice within each adult and immature group. Each experiment was repeated from three to six times.

The levels of IL-6 were evaluated as pg/µg protein of the testicular homogenate. The results are presented as the mean of pg/µg protein ± SEM.

### Statistics

Student's *t* test was used for statistical evaluation, and *p* values below 0.05 were considered significant.

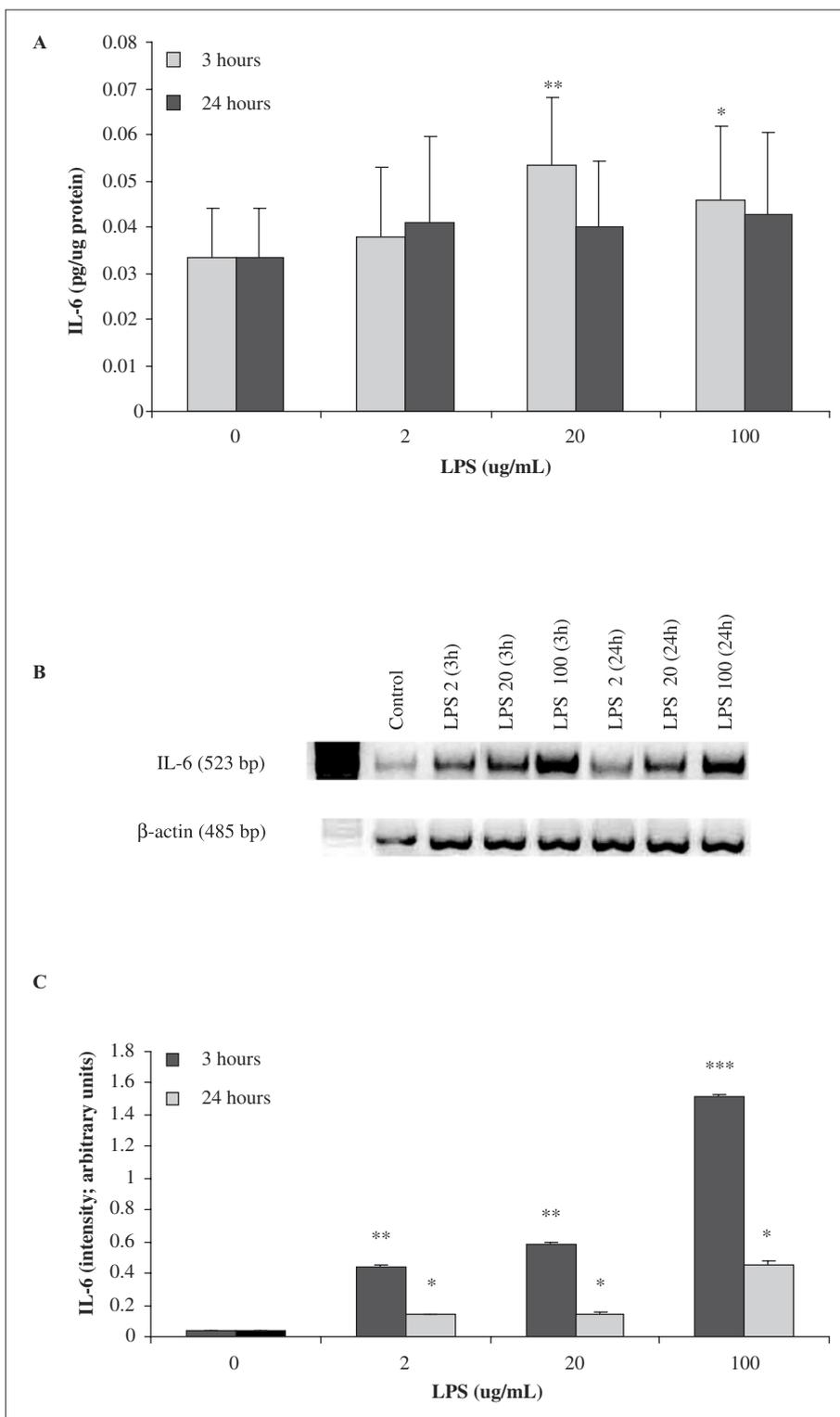
## RESULTS

### Over-expression of IL-6 in testicular homogenates of immature, as compared to mature mice

IL-6 levels in testicular homogenates of immature mice were significantly higher than in mature mice (both protein and RNA levels), before and after LPS injection (*figures 1,2*); the scales of the figures are different (see also our previous paper in the present volume).

### LPS induced the expression of IL-6 in testicular tissues of mature mice

Intraperitoneal injection of LPS into mature mice significantly increased IL-6 levels in their testicular homogenates, in a time- and dose-dependent manner (*figure 1A*). After 3 hours of induction with 20 and 100 µg/mL LPS, the levels of IL-6 in testicular homogenates of adult mice were significantly increased (0.053 ± 0.015 pg/µg protein, *p* = 0.0012 and 0.046 ± 0.016 pg/µg protein, *p* = 0.032 respectively). However, stimulation with 2 µg/mL of LPS for 3 hours did not significantly increase IL-6 levels in testicular homogenates of adult mice (0.038 ± 0.015 pg/µg



**Figure 1**

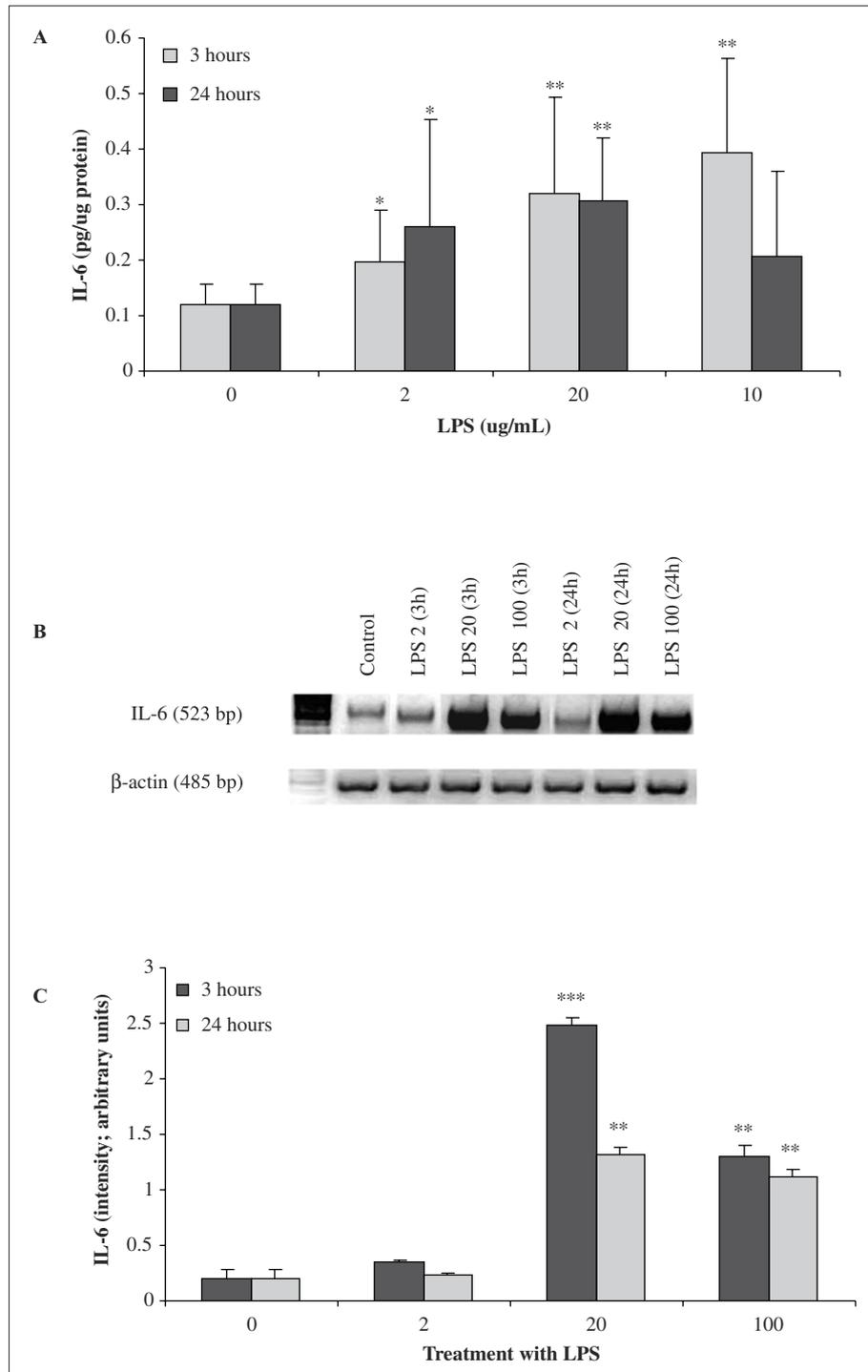
Levels of IL-6 expression induced by LPS in testicular tissues of mature mice.

Mature mice were injected (i.p) with saline (control) or LPS (2, 20, 100 µg/mL) over 3 hours or 24 hours. After each time point, mice were sacrificed and testes were collected and homogenized. IL-6 protein levels were evaluated using a specific ELISA kit. Levels are expressed as pg/µg protein ± SEM (A). The expression levels of IL-6 mRNA were evaluated by RT-PCR using a specific primer, and IL-6 PCR products were separated on 2% agarose gel containing ethidium bromide (B). Quantitative evaluations of the PCR products were performed by video densitometry, and the ratio between IL-6 bands and β-actin bands were calculated (C). The results are expressed as arbitrary units.

\* p < 0.03 \*\* p < 0.005 \*\*\* p < 0.0005.

protein, p = 0.39) as compared to the control group (0.033 ± 0.01 pg/µg protein). Conversely, stimulation of adult mice with various doses of LPS (i.p.) (2, 20 and 100 µg/mL) for 24 hours did not induce significantly

higher levels of IL-6 in testicular homogenates (0.041 ± 0.2 pg/µg protein; 0.04 + 0.014 pg/µg. protein and 0.043 ± 0.018 pg/µg protein respectively), as compared to the control group.



**Figure 2**

Levels of IL-6 expression induced by LPS in testicular tissues of immature mice.

Immature mice were injected with saline (control) or LPS as described in *figure 1A*. IL-6 levels were evaluated using a specific ELISA kit. Levels of the protein are expressed as  $\mu\text{g}/\mu\text{g}$  protein  $\pm$  SEM (**A**). The expression levels and quantification of IL-1 $\beta$  mRNA (**B**, **C**) described in *figure 2 B*, **C** were evaluated using specific primers for IL-6.

\*  $p < 0.04$  \*\* $p < 0.005$  \*\*\*  $p < 0.0003$ .

IL-6 mRNA expression was significantly increased after 3 and 24 hours of induction with various concentrations of LPS (2, 20 and 100  $\mu\text{g}/\text{mL}$ ;  $p < 0.005$ ,  $p < 0.002$  and  $p < 0.0005$  respectively and  $p < 0.01$ ,  $p < 0.03$  and  $p < 0.03$  respectively) as evaluated by RT-PCR (*figure 1B*) and quantified by densitometry (*figure 1C*).

***LPS induced higher expression of IL-6 in testicular tissues of immature mice***

Intraperitoneal injection of LPS into immature mice induced significantly higher IL-6 levels in their testicular homogenates, in a time- and dose-dependent manner (*figure 2A*).

Stimulation with 2, 20 and 100 µg/mL of LPS for 3 hours significantly increased IL-6 levels in testicular homogenates of immature mice ( $0.2 \pm 0.09$  pg/µg protein,  $p = 0.039$ ;  $0.32 \pm 0.17$  pg/µg protein,  $p = 0.008$  and  $0.4 \pm 0.17$  pg/µg protein,  $p = 0.001$  respectively) as compared to the control group ( $0.119 \pm 0.04$  pg/µg protein). Also, the levels of IL-6 in testicular homogenates of immature mice were significantly increased after 24 hours of induction with 2 and 20 µg/mL LPS ( $0.26 \pm 0.19$  pg/µg protein,  $p = 0.036$  and  $0.31 \pm 0.12$  pg/µg protein,  $p = 0.001$  respectively), but not with 100 µg/mL LPS ( $0.21 \pm 0.15$  pg/µg protein,  $p = 0.07$ ).

IL-6 mRNA expression was significantly increased after 3 and 24 hours of induction with various concentrations of LPS (20 and 100 µg/mL;  $p < 0.0003$  and  $p < 0.005$  respectively and  $p < 0.002$ , and  $p < 0.003$  respectively), as evaluated by RT-PCR (figure 2B), and quantified by densitometry (figure 2C).

## DISCUSSION

The cellular origins of IL-6 in the testis, under normal conditions, involve all testicular compartments (see our paper published in the present volume). Systemic inflammation, by i.p. injection of LPS, could induce testicular cells to produce IL-6. It was demonstrated that LPS induced testicular cells to secrete TNF, IL-1 and IL-6 *in vitro* [4, 14, 24, 25]. Also, IL-1 and TNF- $\alpha$  induced testicular macrophages, Sertoli cells and Leydig cells to secrete IL-6 *in vitro* [4, 14, 25].

Systemic infection and inflammatory disease were found to disrupt germ cell production and to impair the reproductive capacity by decreasing steroidogenesis and testosterone levels [6-10, 14]. It was shown that TNF, IL-1 and IL-6 decreased testosterone secretion by Leydig cells [4, 14, 26, 27]. These factors also affected transferrin secretion by Sertoli cells [4, 14, 26]. In addition, IL-1 $\alpha$  was demonstrated to induce germ cell proliferation; however, IL-6 inhibited this function [2, 28, 29]. IL-6 may also have potentially adverse effects on male reproductive function, influencing the hypothalamic-pituitary-testicular axis.

Recently, it was shown that LPS is a potent stimulator of Sertoli cell proliferation *in vitro*, and that it can modulate the mitogenic action of FSH on immature Sertoli cells, thus, disturbing adult germ cell production [30].

IL-6 increases during infection/inflammation could inhibit the immune response by inducing immunosuppressive factors such as IL-1ra and sTNF and depressing regulation of TNF- $\alpha$  production [31], thus protecting testicular tissue. Thus, under pathological conditions IL-6 could interfere with male fertility but may also play an important role in protecting testicular tissue.

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## REFERENCES

1. Skinner MK. Cell-cell-interactions in the testis. *Endocr Rev* 1991; 12: 45.
2. De Kretser DM, Loveland KL, Meinhardt A, Simorangkir D, Wreford N. Spermatogenesis. *Hum Reprod* 1999; 13: 1.
3. Weinbauer GF, Nieschlag E. Hormonal control of spermatogenesis. In: de Kretser DM, ed. *Molecular biology of the male reproductive system*. San Diego: Academic press, 1993: 99.
4. Huleihel M, Lunenfeld E. Regulation of spermatogenesis by paracrine/autocrine testicular factors. *Asian J Androl* 2004; 6: 259.
5. Schlatt S, Meinhardt A, Nieschlag E. Paracrine regulation of cellular interactions in the testis: factors in search of a function environment. *Eur J Endocrinol* 1997; 137: 107.
6. Adamopoulos DA, Lawrence DM, Vassilopoulos P, Contoyiannis PA, Swyer GIM. Pituitary-testicular interrelationships in mumps orchitis and other viral infections. *BMJ* 1987; 1: 1177.
7. Buch JP, Havlovec SK. Variation in sperm penetration assay related to viral illness. *Fertil Steril* 1991; 55: 844.
8. Cutolo M, Balleari E, Giush M, Monachesi M, Accardo S. Sex hormone status of male patients with rheumatoid arthritis: evidence of low serum concentrations of testosterone at baseline and after human chorionic gonadotropin stimulation. *Arthr Rheumat* 1988; 31: 1314.
9. Baker HWG. Reproductive effects of non-testicular illness. *Endocrinol Metabol Clin North Am* 1998; 27: 831.
10. O'Bryan MK, Schlatt S, Phillips DJ, de Kretser DM, Hedger MP. Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels in vivo. *Endocrinology* 2000; 141: 238.
11. Refojo D, Arias P, Moguilevsky JA, Feleder C. Effect of bacterial Endotoxin on in vivo pulsatile gonadotrophin secretion in adult male rats. *Neuroendocrinology* 1998; 67: 275.
12. Dinarello CA. Biologic basis for interleukin-1 in disease. *Blood* 1996; 87: 2095.
13. Imura H, Fukata J, Mori T. Cytokines and endocrine function: an interaction between the immune and neuroendocrine system. *Clin Endocrinol (Oxf)* 1991; 35: 107.
14. Diemer T, Hales DB, Weinder W. Immune-endocrine interactions and Leydig cell function: the role of cytokines. *Andrology* 2003; 35: 55.
15. Syed V, Gerard N, Kaipia A, Bardin CW, Parvinen M, Jegou B. Identification, ontogeny and regulation of an interleukin-6 (IL-6) factor in the rat testis. *Endocrinology* 1993; 132: 293.
16. Syed V, Stephan JP, Gerard N, Legrand A, Parvinen M, Bardin CW, et al. Residual bodies activate Sertoli cell interleukin-1a (IL-1a) release, which triggers IL-6 production by an autocrine mechanism, through the lipoxygenase pathway. *Endocrinology* 1995; 136: 3070.
17. Stephan JP, Syed V, Jegou B. Regulation of Sertoli cell IL-1 and IL-6 production in vitro. *Mol Cell Endocrinol* 1997; 134: 109.
18. Boockfor FR, Schwarz LK. Effects of interleukin-6, interleukin-2 and tumor necrosis factor alpha on transferrin release from Sertoli cells in culture. *Endocrinology* 1991; 129: 256.
19. Cudicini C, Lejeune H, Gomez E, Bosmans E, Ballet F, Saez J, et al. Human Leydig Cells and Sertoli Cells are Producers of Interleukin-1 and -6. *J Clin Endocr Met* 1997; 82: 1426.
20. Kern S, Robertson SA, Mau VJ, Maddocks S. Cytokine secretion by macrophages in the rat testis. *Biol Reprod* 1995; 53: 1407.
21. Hakovirta H, Syed V, Jegou B, Parvinen M. Function of interleukin-6 as an inhibitor of meiotic DNA synthesis in the rat seminiferous epithelium. *Mol Cell Endocrinol* 1995; 108: 193.
22. Hales DB, Diemer T, Held-Hales K. Role of cytokines in testicular function. *Endocrinology* 1999; 10: 201.
23. Lunenfeld B, Weissenberg R. In: Prunty FTG, Gardiner-Hill H, eds. *Modern trends in endocrinology-4*. Trowbridge & London: Redwood press Ltd, 1972: 157.

24. Huleihel M, Lunenfeld E, Horowitz S, Levy A, Poashnik G, Glezerman M. Involvement of serum and LPS in the production of interleukin-1- and interleukin-6-like molecules by human sperm cells. *Am J Reprod Immunol* 2000; 43: 41.
25. Hedger MP, Meinhardt A. Cytokines and the immune testicular axis. *J Reprod Immunol* 2003; 58: 1.
26. Huleihel M, Zeyse D, Lunenfeld E, Zeyse M, Mazor M. Induction of transferrin secretion in murine Sertoli cells by FSH and IL-1; the possibility of different mechanism(s) of regulation. *Am J Reprod Immunol* 2002; 47: 112.
27. Bornstein SR, Rutkowski H, Vrezas I. Cytokines and steroidogenesis. *Mol Cell Endocrinol* 2004; 215: 135.
28. Parvinen M, Soder O, Mali P, Froysa B, Ritzen EM. *In vitro* stimulation of stage-specific deoxyribonucleic acid synthesis in rat seminiferous tubule segments by interleukin-1a. *Endocrinology* 1991; 129: 1614.
29. Pollanen P, Soder O, Parvinen M. Interleukin-1a stimulation of spermatogonial proliferation in vivo. *Reprod Fertil Dev* 1989; 1: 85.
30. Peterson C, Froysa B, Soder O. Endotoxin and proinflammatory cytokines modulate Sertoli cell proliferation in vitro. *J Reprod Immunol* 2004; 61: 13.
31. Tilg H, Dinarello CA, Mier JW. IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. *Immunol Today* 1997; 18: 428.