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Seminal and molecular evidence that sauna exposure affects human spermatogenesis

Andrea Garolla, Mario Torino, Barbara Sartini, Ilaria Cosci, Cristina Patassini, Umberto Carraro, and Carlo Foresta*

Department of Molecular Medicine, Section of Clinical Pathology and Pathology of Human Reproduction Unit, University of Padova, Via Gabelli 63, Padova 35121, Italy

*Correspondence address: Tel: +39-049-8212639; Fax: +39-049-8218520; E-mail carlo.foresta@unipd.it

STUDY QUESTION: What are the effects of continuous sauna exposure on seminal parameters, sperm chromatin, sperm apoptosis and expression of genes involved in heat stress and hypoxia?

SUMMARY ANSWER: Scrotal hyperthermia by exposure to sauna can induce a significant alteration of spermatogenesis.

WHAT IS KNOWN ALREADY: Several authors have evidenced that high temperature has dramatic effects on spermatogenesis.

STUDY DESIGN, SIZE AND DURATION: A longitudinal time-course study. Data from 10 subjects exposed to Finnish sauna were collected before sauna (T0), after 3 months of sauna sessions (T1) and after 3 (T2) and 6 months (T3) from the end of sauna exposure.

PARTICIPANTS/MATERIALS, SETTING AND METHODS: Ten normozoospermic volunteers underwent two sauna sessions per week for 3 months, at 80–90°C, each lasting 15 min. Sex hormones, sperm parameters, sperm chromatin structure, sperm apoptosis and expression of genes involved in heat stress and hypoxia were evaluated at the start, at the end of sauna exposure and after 3 and 6 months from sauna discontinuation. Student's *t*-test for paired data was used for statistical analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: At the end of sauna exposure, we found a strong impairment of sperm count and motility ($P < 0.001$), while no significant change in sex hormones was present. Decreases in the percentage of sperm with normal histone-protamine substitution (78.7 ± 4.5 versus 69.0 ± 4.1), chromatin condensation (70.7 ± 4.7 versus 63.6 ± 3.3) and mitochondrial function (76.8 ± 4.9 versus 54.0 ± 6.1) were also evident at T1, and strong parallel up-regulation of genes involved in response to heat stress and hypoxia was found. All these effects were completely reversed at T3.

LIMITATIONS AND REASONS FOR CAUTION: Absence of subjects with abnormal sperm parameters was the major limitation of this study.

WIDER IMPLICATIONS OF THE FINDINGS: Our data demonstrated for the first time that in normozoospermic subjects, sauna exposure induces a significant but reversible impairment of spermatogenesis, including alteration of sperm parameters, mitochondrial function and sperm DNA packaging. The large use of Finnish sauna in Nordic countries and its growing use in other parts of the world make it important to consider the impact of this lifestyle choice on men's fertility.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was sought for this study and the authors have no conflict of interest to declare.

Key words: heat shock factors / male infertility / sauna / testicular heating / impaired spermatogenesis

Introduction

Spermatogenesis is a complex, multi-step process involving the proliferation and differentiation of spermatogonia into mature sperm. In most mammals testes need to be 2–8°C below core body temperature to work their best and in most species they reside outside the main body cavity (Ivell, 2007). Many studies have shown that elevated intratesticular temperature has an adverse effect on spermatogenesis and that extrinsic thermal stress to the scrotum may cause alteration of sperm parameters and apoptosis (Mieusset

et al., 1991; Mieusset and Bujan, 1995; Jung and Schuppe, 2007). Previous findings in animal models have suggested that altered spermatogenesis secondary exposure of the testis to high temperature could be caused by insufficient blood supply and consequent hypoxic stress at parenchymal cells (Galil and Setchell, 1988; Setchell, 1998). In humans scrotal heating of 1–1.5°C above normal has been shown to induce reduction in testis size, decreased sperm production and altered sperm morphology (Bedford et al., 1982; Jung and Schuppe, 2007). It is well known that some clinical disorders such as cryptorchidism and varicocele can be involved in the pathophysiology of impaired spermatogenesis by inducing testicular blood flow alteration, hyperthermia and thus hypoxia and oxidative stress (Mieusset et al., 1987; Fretz and Sandlow, 2002). Moreover, it has been documented that scrotal heating activates testicular stress responses common to other tissues such as: (a) expression of hypoxia-inducible factor 1 alpha (HIF1α), a transcription factor whose target genes are involved in vasodilation, angiogenesis and glycolysis (Shweiki et al., 1992; Wenger, 2002; Paul et al., 2009); (b) HIF1α induced gene expression of vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis and endothelial growth (Forsythe et al., 1996) acting through interaction with its respective receptors: the kinase insert domain (KDR) and the fms-related tyrosine kinase 1 (FLT1) (Ferrara, 2004) and (c) activation of heat shock proteins (HSPs), which are in turn regulated by heat shock factors (HSFs; Feder and Hofmann, 1999). HSPs are described as chaperonins with protective and antiapoptotic roles, assisting the correct folding of nascent and stress-accumulated misfolded proteins and preventing their aggregation in response to thermal and oxidative stress. Increased expression of HSPs and HSFs in sperm from oligozoospermic men has been generally considered a testicular attempt to repair spermatogenic impairment and germ cell damage (Katschinski, 2004; Lima et al., 2006; Lanneau et al., 2008). A previous study by our group (Ferlin et al., 2010) showed that HSPs and HSFs are expressed in ejaculated sperm at different levels. In particular, we demonstrated that these proteins are up-regulated in patients with oligozoospermia and varicocele, suggesting that this may represent a molecular marker of response to testicular damage. Recently, there has been growing interest in the hypothesis that certain environmental conditions, lifestyles and occupational habits may impair male reproductive health and could be responsible for male infertility by inducing testicular heating (Jung and Schuppe, 2007). Moreover, it has been documented that prolonged exposure to sauna may affect male fertility (Procope', 1965; Brown-Woodman et al., 1984; Saikhun et al., 1998); however, existing data are not conclusive. In this study, we evaluated the effects of thermal stress induced by the use of a standard programme of sauna sessions on reproductive hormones, sperm parameters and apoptosis; furthermore, we studied the testicular expression of HIF-1α, VEGF and its respective receptors KDR and FLT1, as well as HSPs and HSFs.

Materials and Methods

Patients, sauna programme and sampling

This study was approved by our Local Ethics Committee. Ten normozoospermic, healthy volunteers (mean age 33.2 ± 4.7 years) were recruited in the study after obtaining informed consent for the use of sperm for research purposes. The exclusion criteria were: varicocele, metabolic syndrome, malignancies, history of cryptorchidism, sexual hormone alteration and sauna exposure during the previous year. The Finnish sauna programme consisted of full body exposure to 80–90°C with humidity level ranging from 20 to 30% for 15 min, 2 days per week for 3 months. Scrotal temperature was recorded with infrared thermometer (Kramer Electronics, Italy). The bulb was placed on the scrotal skin between the two testes and the testes pushed together until the temperature was stable. Temperature measurements were taken on the subjects before and immediately after each sauna session. Each measurement was repeated three times and the mean value used. All the participants underwent blood withdrawal and semen analysis (with 2–5 days of sexual abstinence) at each point of the study: before starting the sauna programme (T0), after 3 months of sauna sessions (T1), after 3 (T2) and 6 months (T3) from the end of the sauna exposure.

Semen sample collection and processing

Semen samples were obtained from each subject by masturbation after 3 days of sexual abstinence and collected in sterile containers. Samples were allowed to liquefy for 30 min and were examined for semen volume, pH, sperm concentration, viability and normal morphology according to the World Health Organization guidelines for the examination and processing of human semen (WHO, 2010). A duplicate reading was performed by different operators and the results are the mean of these determinations. All the samples had normal viscosity and did not contain leukocytes. Round cells were counted by using the improved Neubauer haemocytometer and the presence of leukocytes was assessed in the semen smeared and stained with the Papanicolaou procedure. Semen culture was negative and antisperm antibodies were absent in all the subjects.

Hormone assays

Plasma concentrations of FSH and LH were measured in each subject by radioimmunoassay using ^{125}I-labeled FSH and LH (Ares-Serono, Milan, Italy). Testosterone plasma levels were determined in all the subjects using the double antibody radioimmunoassay utilizing commercial kits (Radim, Rome, Italy). Inhibin plasma concentrations were measured by solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) specific to the dimeric inhibin-B form (Serotec, Oxford, UK).
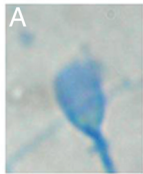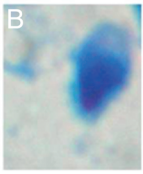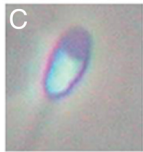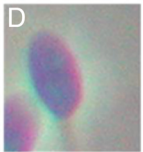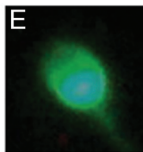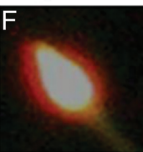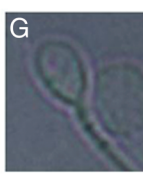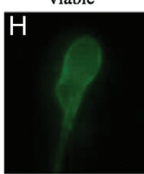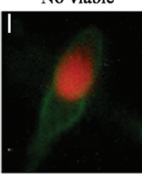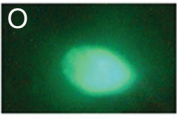
Analysis of sperm chromatin condensation and stability

Aniline test

For the evaluation of histone replacement by sperm-specific nuclear protamines, semen samples were washed in 0.2 M phosphate buffer (pH 7.2) and fixed in 3% glutaraldehyde for 30 min before air drying sperm smears on glass slides (Fig. 1A and B). The sperm smears were then stained for 5 min in 5% aniline blue in 4% acetic acid (pH 3.5) as previously described by other authors (Hofmann and Hilscher, 1991). Sperm were classified as either blue stained (immature nucleus due to histone persistence, Fig. 1A) or unstained (mature nucle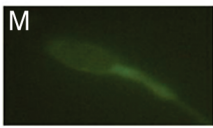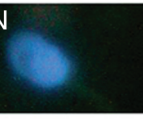us with complete histone-protamine replacement, Fig. 1B) by counting 200 spermatozoa per slide. Results were expressed as the percentage of spermatozoa that were mature (unstained).

Decondensation test

Chromatin stability was evaluated as previously described (Huret, 1983) by inducing decondensation by incubation with detergent and chelating agents (SDS/EDTA 5%) for 15 min at 37°C (Fig. 1B and C). Spermatozoa whose nuclear chromatin had decondensed could be recognized by having a swollen opaque head with reduced light refractivity, under

	Normal	Pathologic
Protamination (*ANILINE BLUE*)	A	B
DNA condensation (*SDS*)	C	D
Chromatin integrity (*ACRIDINE ORANGE*)	Double strand E	Single strand F
Phosphatidylserine externalization (*ANNEXIN-V*)	G	Viable H No viable I
Mitocondrial function (*JC-1*)	L	M
DNA fragmentation (*TUNEL*)	N	O

Figure 1 Evaluation of sperm histone-protamine substitution by aniline staining (**A** and **B**), DNA condensation by SDS test (**C** and **D**), chromatin integrity by AO staining (**E** and **F**), phosphatidilserine externalization by Annexin-V and Propidium iodide (**G**–**I**), mitochondrial function by JC1 test (**L** and **M**) and DNA fragmentation by terminal deoxynucleotidyl TUNEL assay (**N** and **O**). (A) Sperm cell with normal protamination; (B) cell with abnormal histone persistence; (C) sperm with normally condensed DNA; (D) cell with decondensed DNA; (E) sperm with normal DNA integrity; (F) sperm cell with altered DNA integrity due to the presence of single-strand DNA; (G) viable non-apoptotic sperm; (H) viable cell with phosphatidilserine externalization; (l) dead cell; (L) Sperm cell with active mitochondrial function; (M) cell with mitochondrial membrane potential alteration; (N) cell with 'native' DNA; (O) cell with DNA fragmentation.

phase-contrast microscopy. Where the nuclear chromatin did not decondense, the spermatozoa had a normal appearance (Fig. 1C). Results were expressed as the percentage of sperm that exhibited a normal appearance (Fig. 1D) based on counting 200 spermatozoa per slide.

Acridine orange test

The acridine orange (AO) test was performed as previously reported by Egger-Kruse *et al*. (1996). The AO test (Fig. 1E and F) was used in order to assess the integrity of sperm DNA. Briefly, equal volume samples of human semen and AO solution were mixed together on the surface of a glass slide and covered with a glass coverslip. The sample was then evaluated with a fluorescence microscope with a 490 nm

excitation light and 530 nm barrier filter. Nuclei from 100 spermatozoa were examined and scored as with green- or red-fluorescence. When the head showed green fluorescence (Fig. 1E), sperm were considered normal (double-stranded DNA); they were considered denatured (Fig. 1F) when fluorescence was red (single-stranded DNA). Results were expressed as the percentage sperm that showed normal (green) fluorescence.

Annexin-V/PI assay

The Annexin-V/PI assay was performed as previously described (Barroso *et al*., 2000). Annexin-V-fluorescein isothiocyanate (FITC) staining (Fig. 1G–I) was assessed by Apoptosis Detection Kit (BD Biosciences,

San Jose, CA, USA). Spermatozoa stained by both propidium iodide and annexin-V were considered dead (Fig. 1I); only those stained with annexin-V were considered apoptotic (Fig. 1H), whereas those with no staining were considered viable and non-apoptotic (Fig. 1G). Results were expressed as the percentage of sperm that were apoptotic.

Assessment of sperm mitochondrial status

Assessment of sperm mitochondrial status was performed as previously reported (Gillan et al., 2005). Sperm mitochondrial activity was evaluated by staining with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenimidazolyl carbocyanine iodide, Fig. 1L and M) according to the manufacturer's procedure (Invitrogen, Molecular Probes, Eugene, OR, USA). JC-1 reversibly shifts its fluorescence emission from green (monomeric status) to orange (multimeric status) with the increase of mitochondrial membrane potential. Cells with high fluorescence emission for both green and orange were considered to be featured by normal mitochondrial status (Fig. 1L), whereas cells featured by the sole green fluorescence were considered to have impaired mitochondrial function (Fig. 1M). Results were expressed as the percentage sperm that exhibited abnormal function.

Evaluation of DNA fragmentation

Evaluation of DNA fragmentation was performed as previously reported by Tesarik et al. (2004). The presence of sperm apoptosis related to DNA strand breaks was evaluated by terminal deoxynucleotyl transferase-mediated d'UTP nick-end labelling (TUNEL), by means of the 'In situ cell detection kit' (Roche Diagnostics GmbH, Germany) with FITC-labelled d'UTP (Fig. 1N and O). For each sample, 200 spermatozoa were evaluated through the fluorescent microscope with a $100\times$ oil immersion objective. Results were expressed as the percentage of cells with fragmented DNA (green staining, Fig. 1O) versus normal cells (blue staining, Fig. 1N).

RNA extraction, cDNA synthesis and RT–PCR

RNA Extraction, cDNA Synthesis and RT–PCR were performed as previously reported by Östling et al. (2007). Sperm pellet was obtained after washing native semen samples twice with PBS and total RNA was extracted from sperm pellets using the RNA-spin™ Mini kit. All the isolated RNAs were quantified by determining the ratio of optical density at 260/280 nm with a Nano-Drop® spectrophotometer. Total cDNA was amplified by PCR using specific primers for each gene to verify sperm cDNA presence and quality. As a negative control, cDNA was omitted.

The sequence of primer couples for the following gene panel: HSP90, HSP70, HSF1, HSF2, HSFY, HIF-1α, VEGF, FLT1 are reported in Table I.

Quantitative real-Time PCR

Quantitative Real-Time PCR was performed as previously described (Cedenho et al., 2006). Gene expression in ejaculated sperm was quantified by real-time PCR using the Bio-Rad iQ™5 system according to the manufacturer's instructions with SYBR® Green PCR Master Mix. Amplification reactions were performed in a 25 μl final volume containing 12.5 μl Power SYBR Green PCR Master Mix, 1 μl primers (10 μM) and 4 μl (20 ng) cDNA from ejaculated sperm. Amplification was performed for 45 cycles. After an initial warming for 10 min, each cycle consisted of 15-s denaturation, 30-s annealing at 60°C and 30-s extension at 60°C. To calculate data, we used the comparative Ct method for relative quantification (ΔΔCt), which describes the change in expression of the target gene in the tested sample relative to a calibrator sample from a cDNA library and provides accurate comparison between the initial level of

Table I Sequences of primers used for the following gene panel: *HSP90, HSP70, HSF1, HSF2, HSFY, HIF-1α, VEGF, FLT1* and *KDR*.

Gene	Primers
β-Actin	Forward 5′-CACTCTTCCAGCCTTCCTTCC-3′
	Reverse 5′-CGGACTCGTCATACTCCTGCT-3′
GAPDH	Forward 5′-AAGGTGAAGGTCGGAGTCAA-3′
	Reverse 5′-AATGAAGGGGTCATTGATGG-3′
HSP90	Forward 5′-ACAGGTGAGACCAAGGACCA-3′
	Reverse 5′-CGGTTTGACACAACCACCTT-3′
HSP70	Forward 5′-ATGAGTATAGCGACCGCTGC-3′
	Reverse 5′-TCCTTGGACTGTGTTCTTTGC-3′
HSF1	Forward 5′-GCCTTCCTGACCAAGCTGT-3′
	Reverse 5′-GTCGAACACGTGGAAGCTGT-3′
HSF2	Forward 5′-ACGAGTTCATCACCTGGAGC-3′
	Reverse 5′-GCCTCACAAAGCTTGCCATA-3′
HSFY	Forward 5′-TTTTGTTCGACAGCTCAACCT-3′
	Reverse 5′-CCACGCTTGAAATTTGGATT-3′
HIF1α	Forward 5′-GAAGACATCGCGGGGAC-3′
	Reverse 5′-TGGCTGCATCTCGAGACTTT-3′
VEGF	Forward 5′-CCCACTGAGGAGTCCAACAT-3′
	Reverse 5′-TTTCTTGCGCTTTCGTTTTT-3′
FLT1	Forward 5′-AAGAAGGAAACAGAATCTGCAA-3′
	Reverse 5′-TCCCTTCCTTCAGTCATGTGT-3′
KDR	Forward 5′-CCTGTATGGAGGAGGAGGAA-3′
	Reverse 5′-CGGCTCTTTCGCTTACTGTT-3′

template in each sample. Data were analysed with iQ5 2.0, version 2.0.148.060623 standard edition optical system software.

Statistical analysis

All results are expressed as the mean value ± standard deviation of the mean of three independent experiments. Data were compared using Student's t-test for paired data. P-values <0.05 were considered to be statistically significant.

Results

The mean scrotal temperatures before and after the sauna sessions were $34.5 \pm 0.6°C$ and $37.5 \pm 0.4°C$, respectively ($P < 0.01$). Figure 2 reports the mean sperm parameters observed in 10 normozoospermic subjects before (T0), after 3 months of sauna exposure (T1) and after 3 (T2) and 6 months (T3) from the end of the sauna programme. Figure 2A shows that both sperm concentration (10^6/ml) and count (10^6) were significantly reduced at the end of the sauna programme compared with baseline (respectively, 31 ± 13.1 versus 89 ± 29.3 and 93 ± 27.0 versus 223 ± 52.8, both $P < 0.001$). Three months after the end of the sauna programme (T2), a reduced total sperm count was still present (138.4 ± 48.6, $P < 0.01$), while at T3 this parameter was completely restored. When considering other sperm parameters, we found a reduced percentage of progressive motile sperm at the end of sauna exposure (36.1 ± 3.6
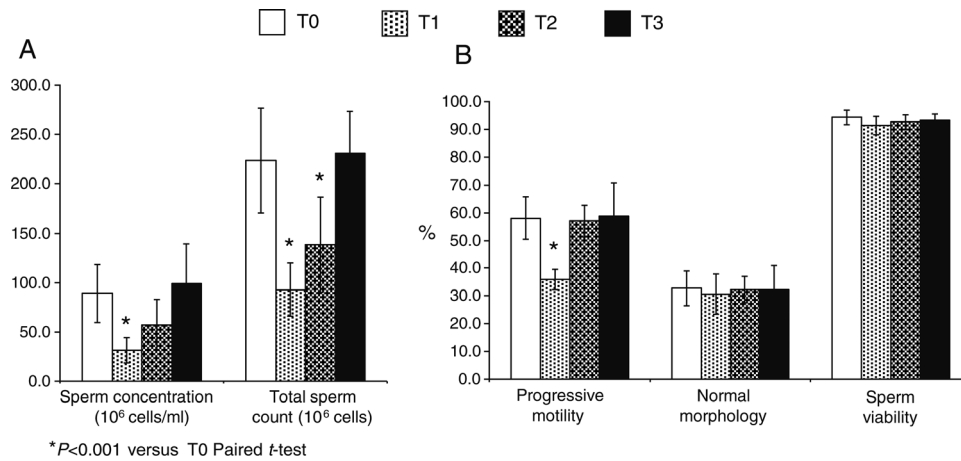
Figure 2 Evaluation of sperm parameters during the whole study period: T0 = before sauna exposure, T1 = after 3 months of sauna sessions, T2 = after 3 months and T3 = after 6 months from the end of sauna exposure. Data are means ± SD, $n = 10$.

Table II Mean sex hormone concentrations observed in normozoospermic patients before (T0), after 3 months of sauna sessions (T1) and after 3 (T2) and 6 months (T3) from the end of sauna exposure.

|	FSH (U/l)	LH (U/l)	T (nmol/l)	E2 (pmol/l)	InhB (pg/l)	SHBG (nmol/l)
T0	4.1 ± 1.3	6.1 ± 2.8	20.0 ± 6.3	109.3 ± 22.2	121.3 ± 43.3	26.2 ± 11.7
T1	4.0 ± 0.9	5.5 ± 1.4	20.7 ± 4.9	109.2 ± 27.2	106.2 ± 51.4	26.3 ± 11.0
T2	4.0 ± 1.3	5.6 ± 2.4	17.4 ± 4.8	87.7 ± 26.8	101.7 ± 41.8	27.5 ± 12.1
T3	4.1 ± 1.1	5.0 ± 1.3	16.3 ± 4.1	90.7 ± 16.8	117.3 ± 32.4	26.0 ± 11.9

All $P > 0.05$ paired t-test.
Data are means ± SD, $n = 10$.

and 58.0 ± 7.6 comparing T1 versus T0, $P < 0.01$), whereas semen volume, sperm morphology and viability did not change during the whole study period (Fig. 2B). Table II shows the mean plasma concentrations of FSH, LH, testosterone, estradiol, inhibin-B and sex hormone-binding globulin (SHBG) observed at different times of the study; No significant variation of any hormone was observed during the whole study. To evaluate the sperm DNA status, we used many tests whose results are shown in Figure 3. Chromatin packaging was assessed by AO staining, the aniline test and the sperm decondensation assay (Fig. 3A). The AO test showed no significant variation of sperm DNA integrity after sauna induced heat stress. On the contrary, histone-protamine replacement and chromatin condensation (tested by aniline and decondensation tests) displayed a significant impairment at T1 compared with T0 69.0 ± 4.1 versus 78.7 ± 4.5 and 63.6 ± 3.3 versus 70.7 ± 4.7(respectively, both $P < 0.05$). These chromatin alterations completely disappeared at 3 months from the end of the sauna exposure. At the same time, the evaluation of molecular markers of early and late sperm apoptosis (Fig. 3B) documented a significant increase in the percentage of sperm with impaired mitochondrial function compared with basal both at the end of the sauna exposure and up to 3 months from sauna suspension (76.8 ± 4.9 versus 54.0 ± 6.1 and 71.2 ± 4.3 respectively, $P < 0.01$ and $P < 0.05$). In contrast, markers of later stage apoptosis (Annexin-V and

TUNEL tests) showed no significant variation during the whole period, despite an apparent increase at T1. Gene expression of hypoxia-related factors evaluated in ejaculated spermatozoa at different points within the study is reported in Fig. 4. HIF1α, KDR and FLT1 showed a spiked increase after the end of the sauna programme (T1). Thereafter, at T2 they had a rapid reduction and at T3 HIF1α and FLT1 showed lower levels than T0 ($P < 0.001$ versus T0). Interestingly, VEGF expression displayed a similar but delayed trend with a significant increase at T2 ($P < 0.001$ versus basal) and a prompt reduction at the end of the study period. Gene expression analysis of HSPs and factors observed in ejaculated sperm along the study is shown in Fig. 5. All these genes displayed a significant up-regulation after the sauna exposure (T1). Thereafter, HSP90, HSF1 and HSFY had a swift return to baseline levels after the first trimester of recovery, while HSF2 showed a higher expression up to T2 and HSP70 up to T3. Unlike HIF1α and FLT1, the trend of gene expression of HSPs and HSFs during recovery times never showed lower levels than basal.

Discussion

Several authors have published evidence that both high ambient and scrotal temperatures have dramatic consequences on spermatogenesis (Lue *et al*., 1999; Jung *et al*., 2001, 2002; Lue *et al*., 2002;
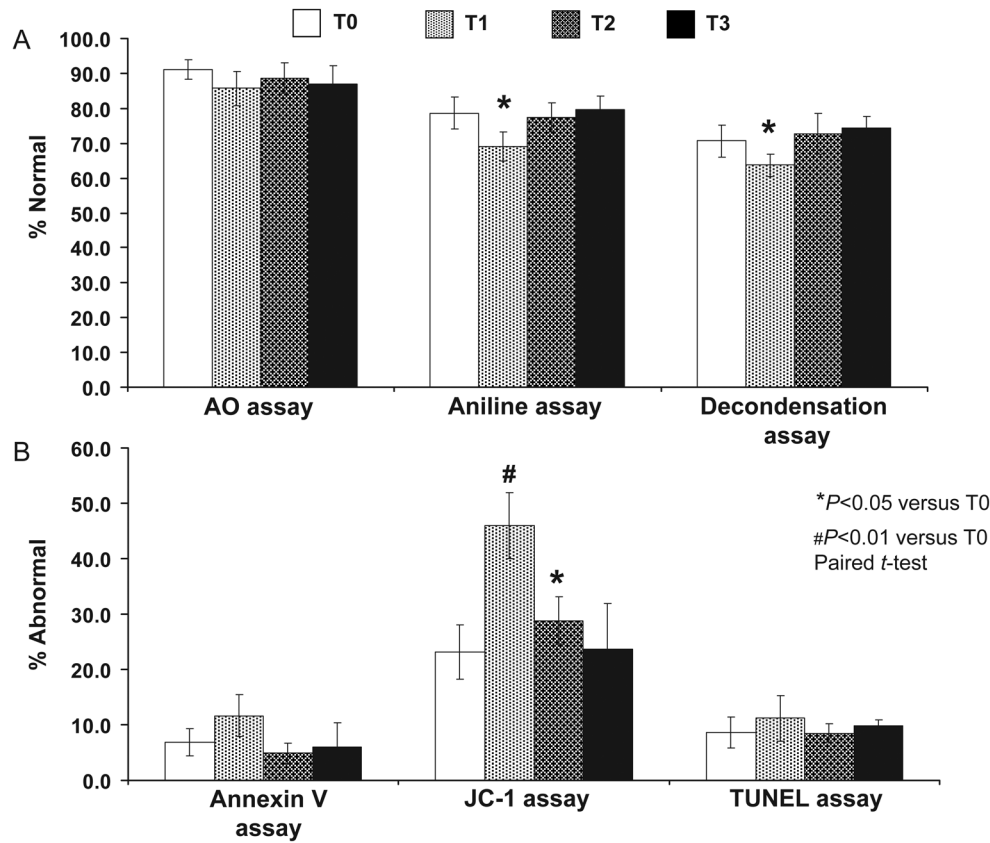
Figure 3 Results of tests evaluating sperm chromatin (**A**) and apoptosis (**B**) at T0 = before sauna exposure, T1 = after 3 months of sauna sessions, T2 = after 3 months and T3 = after 6 months from the end of sauna exposure. (A): normal sperm integrity (AO), histone-protamine replacement (Aniline) and chromatin condensation (Decondensation); (B) phosphatidilserine externalization (A-V), altered mithochondrial functionl (JC-1) and DNA fragmentation (TUNEL). Data are Means ± SD, $n = 10$.
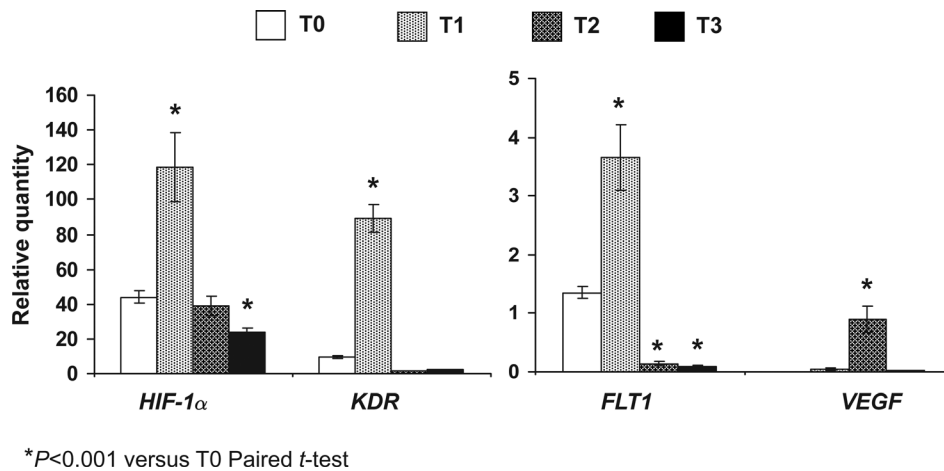
Figure 4 Results of quantitative real-time PCR of HIF1α, VEGF, KDR and FLT1 gene expression performed in ejaculated sperm from subjects evaluated at T0 = before sauna exposure, T1 = after 3 months of sauna sessions, T2 = after 3 months and T3 = after 6 months from the end of sauna exposure. Data are Means ± SD, $n = 10$.
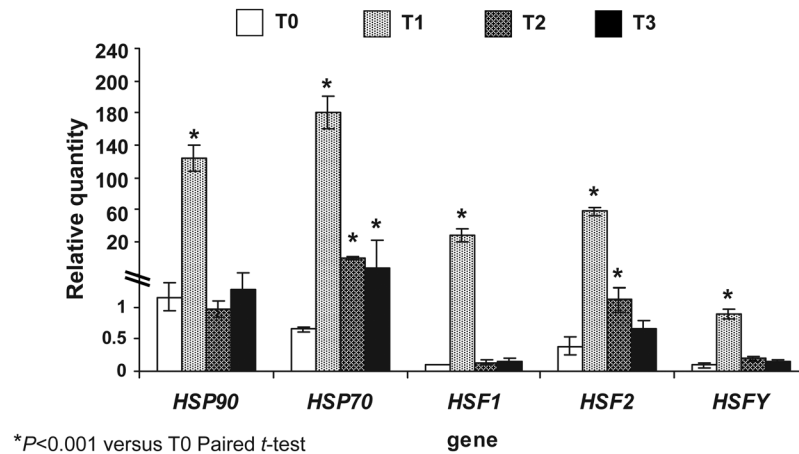
Figure 5 Results of quantitative real-time PCR showing HSPs and factors (HSP90, HSP70, HSF1, HSF2 and HSFY) genes expression in ejaculated sperm from subjects evaluated at T0 = before sauna exposure, T1 = after 3 months of sauna sessions, T2 = after 3 months and T3 = after 6 months from the end of sauna exposure. Data are Means ± SD, n = 10.

Schwalm et al., 2007; Pérez-Crespo et al., 2008; Paul et al., 2008a,b, 2009). However, these authors evaluated the effect of different heat stress ranging from 29 to 43 °C observed in a variety of conditions: whole body, lower third of the body or just scrotal heating, in men and in conscious or anaesthetised animal models. Such large variation in experimental conditions makes it difficult to reach a final conclusion on this topic. In humans, Finnish sauna, which is frequently used for recreational aims, represents a good model of testis hyperthermia (Procope', 1965; Brown-Woodman et al., 1984; Saikhun et al., 1998). Nevertheless, available studies associating sauna and male fertility display ambiguous results, possibly due to the lack of standardized protocols of heat exposure. Some authors have suggested that increasing testicular and body temperature after many sauna sessions induced a decline of sperm count and motility (Procope', 1965; Saikhun et al., 1998). Even acute testicular heat stress at a single sauna session has been reported to impair sperm parameters (Brown-Woodman et al., 1984). In the present study, we evaluated the effects of frequent heat stress induced by sauna sessions at a standardized temperature, humidity level and time of exposure, continued for long enough to overlap an entire spermatogenic cycle. Our results showed a significant reduction of sperm count and progressive motility but no modification of sperm morphology and viability. These modifications were not paralleled by changes of hormone levels (Lue et al., 2002) probably due to the short period of exposure and because spermatogenesis although impaired remained in the fertile range. However, it is not known whether a longer exposure, particularly in subjects with reduced testicular function can impair spermatogenesis up to infertility. Besides changes in sperm count and motility, we found a parallel alteration of DNA protamination and nuclear condensation. This phenomenon has already been investigated in a model of hyperthermia induced by influenza in humans (Evenson et al., 2000), in which sperm from patients with recent episodes of high fever had a pathological protamine-histones replacement. Considering that protamine substitution is responsible for correct sperm DNA condensation and integrity during the latter phases of spermatogenesis, our results seems to confirm previous data reporting that heat stress may induce an alteration of chromatin package during spermiogenesis up to infertility. At the same time, some pathological conditions associated with vascular flow defects at testicular level such as cryptorchidism and varicocele that are frequently associated with histone persistence, hypocondensed sperm DNA and reduced fertility (Foresta et al., 1992; Salsabili et al., 2006). It is notable that pachytene spermatocytes and round spermatids are the testicular cells most sensitive to heat stress (Chowdhury and Steinberger, 1970; Kandeel and Swerdloff, 1988). In fact, when exposed to high temperature they undergo apoptosis via the mitochondrial pathway (Lue et al., 2002; Zhang et al., 2003; Vera et al., 2004). Consistent with this, we found an increased percentage of ejaculated spermatozoa with impaired mitochondrial membrane potential that could explain the reduced sperm motility observed after sauna exposure. These findings suggest that heat stress may induce sperm damage confined to mitochondria; also no significant alteration of latter phases of sperm apoptosis such as phosphatidilserine externalization and DNA fragmentation were found. Therefore, we can hypothesize that the reduction of sperm count observed after heat stress could be related merely to a less efficient spermatogenic process. In general, cell exposure to increased temperature induces the expression of genes involved in the response to hypoxia. In these conditions HIF1α in particular is up-regulated (Wenger, 2002; Paul et al., 2009) and it has been shown to translocate into germ cell nucleus. Besides, it has been demonstrated that HIF1α activates the transcription of VEGF (Shweiki et al., 1992; Forsythe et al., 1996), a factor with fundamental roles in vasculogenesis and angiogenesis (Kim et al., 1993; Carmeliet et al., 1996; Ferrara et al., 1996; Obermair et al., 1997). Moreover, VEGF has been shown to play a major role in decreasing sperm apoptosis during studies on experimentally varicocele-induced rats (Tek et al., 2009). In this study, we observed an increased expression of HIF1α and VEGF's receptors KDR and FLT1, after3 months of sauna exposure and a later increase of VEGF after 3 months from sauna discontinuation. On this basis, we can speculate that at testicular

level, the response to heat stress is initiated by HIF1α activation and thus by VEGF pathway. A protective function in cell survival during otherwise lethal conditions has also been demonstrated for HSPs and their regulating factors HSFs. In fact, these genes act to modulate the correct folding of nascent and stress-accumulated misfolded proteins and prevent their aggregation. In a previous study performed on ejaculated sperm, we demonstrated that these proteins and factors had a higher expression in varicocele and oligozoospermia patients than in sperm from controls (Ferlin *et al.*, 2010). Also, in the present study, we found a strong up-regulation of these genes after heat stress exposure, probably aimed to protect cells from spermatogenic damage. The cause−effect relationship between testicular heat exposure and HSPs and HSFs system activation is clearly shown by its promptly decreased sperm expression when sauna was discontinued. In conclusion, our data demonstrated for the first time that in normozoospermic subjects scrotal hyperthermia induced a significant but reversible impairment of spermatogenesis, including alteration of sperm parameters, mitochondrial function and sperm DNA packaging. Moreover, genital exposure to Finnish sauna resulted in a complex gene response, including expression of genes associated with heat stress and hypoxia. The strong up-regulation of these genes observed in men after sauna exposure suggested that it might represent both a molecular marker of stress response and an attempt to preserve spermatogenesis from testicular heating. It is not clear whether our findings are related to elevation of testicular temperature or to side effects induced by heating of whole body. Whatever, the large use of Finnish sauna in Nordic countries and its growing use in other parts of the world (Hannuksela and Ellahham, 2001; Kluger, 2011) make it important to consider the impact of this lifestyle choice on men's fertility. More studies are needed to understand whether the same protective mechanisms observed in normozoospermic men are fully operational in subfertile men and in prepubertal subjects, in whom spermatogenesis is at a more vulnerable developmental period, and whether they can protect such men from testicular damage.

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Authors' roles

All the authors had full access to the original data, reviewed the data analysis, contributed to data interpretation and to the writing of the report, made final decisions on all parts of the report and approved the final version of the submitted report. A.G., M.T. and C.F. participated in study design and U.C. and C.P. enrolled subjects. M.T. and B.S. undertook statistical analysis. I.C. and C.P. made molecular analysis. A.G., M.T. and B.S. contributed to data collection and generation of tables and figures.

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Conflict of interest

None declared.

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