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In the name of God, most Gracious, most Compassionate

« Nous sommes les enfants d'un monde
Dévasté qui s'essaient à renaître dans
Un monde créer. Apprendre à devenir
Humain est la seule radicalité »

Raoul Vaneigem

« The reward of deeds depends upon the intentions
And every person will get the reward according
to what he has intended »

Muhammad PBUH

« May I become at all times, both now and forever,
A protector for those without protection,
A guide for those who have lost their way,
A ship for those with oceans to cross,
A sanctuary for those in danger,
A lamp for those in the dark,
And a servant to all those in need
As long as living beings exist,
And suffering afflicts them,
May I too abide to dispel the misery of the world »

To

My father Chaudhry Atta-Ullah Baryar (may his soul rest in peace)

Sughran, my mother, who dedicated her whole life for her kids

Huma, my wife, for great patience, oceans away, waiting for me to finish this task

My brothers, sisters and my family for all the rich moments

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RESUME

La spermatogenèse chez l'homme et chez les autres mammifères nécessite une température inférieure à la température du corps. Ainsi, une augmentation de la température des testicules produit des effets délétères sur la fertilité masculine. Une réduction de la concentration spermatique, de la mobilité, de la vitalité et de la morphologie des spermatozoïdes a été rapportée chez les animaux et les hommes lorsque le scrotum ou le corps étaient exposés à des températures plus élevées. Dans les modèles animaux, des températures supérieures à la normale entraîneraient des altérations de l'intégrité de la chromatine des spermatozoïdes conduisant à un retard du développement embryonnaire précoce, à une réduction du taux de grossesse et à des fausses couches. Dans ce contexte, nous avons étudié les effets d'une légère augmentation (+2 °C) de la température des testicules et des épididymes sur l'intégrité de la chromatine des spermatozoïdes chez les hommes.

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Les résultats du SCSA ont montré une augmentation significative du pourcentage de DFI et du pourcentage de HDS du sperme respectivement dès 20 jours (J20) et J34 d'hyperthermie. Les taux de DFI et de HDS sont restés plus élevés pendant les 120 jours d'hyperthermie comparés aux valeurs avant augmentation de la température (valeurs contrôles). Le test du bleu d'aniline a montré une augmentation non significative du pourcentage de spermatozoïdes avec une chromatine immature à J34 d'hyperthermie, atteignant une valeur significative à J73. Cette augmentation est demeurée constante pendant toute la durée d'hyperthermie traduisant un pourcentage de spermatozoïdes avec une chromatine immature supérieur à celui évalué avant hyperthermie. Après l'arrêt de l'hyperthermie, le DFI et le HDS sont revenus à leurs valeurs pré-exposition plus précocement (J73) que le pourcentage de spermatozoïdes avec une chromatine immature (J180).

L'indice d'anomalies multiples (IAM) des spermatozoïdes est augmenté dès J9. La mobilité, la numération et la vitalité des spermatozoïdes ont été significativement diminuées par rapport aux valeurs avant hyperthermie, respectivement à partir de J20, J34 et J34 et sont restées diminuées pendant toute la durée de l'hyperthermie. Après l'arrêt de l'hyperthermie, tous les paramètres spermatiques sont revenus aux valeurs contrôles à partir de J73.

Nous avons montré, pour la première fois chez l'homme, qu'une légère augmentation de la température des testicules et des épididymes a un effet sur l'intégrité de la chromatine du spermatozoïde avant même la baisse de la numération des spermatozoïdes. Compte tenu des résultats obtenus dans les modèles animaux, les résultats de ce protocole expérimental pose la question des conséquences possibles de l'altération de la chromatine sur le développement embryonnaire alors que les paramètres du sperme ne sont pas incompatibles avec une fécondation au cours de la contraception masculine, durant les phases de récupération et d'inhibition. De plus, nos résultats pourraient être d'intérêt en infertilité masculine, en assistance médicale à la procréation et dans les fausses couches à répétition.

Mots clés: spermatozoïdes, fragmentation de l'ADN, hyperthermie, SCSA, épididyme, température testiculaire, contraception masculine, chromatine du spermatozoïde.

ABSTRACT

In man and other mammals, spermatogenesis is ensured at a temperature lower to body temperature. An increase in temperature has deleterious effects on male fertility. Reduced sperm out put, motility, viability and morphology have been reported in animals and men at higher than normal scrotal or body temperatures. Increased temperature caused alterations in sperm chromatin integrity resulting in retarded early embryo development, reduced pregnancy rates and miscarriages in animal models. In this context, for the first time in men, we investigated the effects of a mild increase (+2 °C) in testes and epididymal temperature on sperm chromatin integrity.

We designed an experimental protocol to induce mild testes and epididymal hyperthermia in five healthy fertile volunteers. Testes were lifted up and maintained at supra scrotal position 15 hours daily for 120 consecutive days. Classical sperm parameters were assessed according to World Health Organization (WHO) guidlines. Sperm DNA fragmentation index (DFI) and high DNA stainability (HDS) were analysed by "Sperm Chromatin Structure Assay" (SCSA) and acid aniline blue staining technique was used to assess the sperm chromatin maturity.

The results of SCSA showed a significant increase in the percentage of sperm DFI and HDS as early as day 20 (D20) and D34 respectively during hyperthermia which remained higher compared with the values before hyperthermia (controls) during the entire period of hyperthermia. Aniline blue test showed a non-significant increase in the percentage of sperm with immature chromatin as early as D34 during hyperthermia reaching significance at D73 and remained higher than control during the entire period of hyperthermia. After cessation of hyperthermia sperm DFI and HDS returned to the respective control values earlier (D73) than the percentage of aniline blue positive sperm (D180).

Multiple anomalies index (MAI) of spermaozoa was significantly increased, compared to control, as early as D9 of hyperthermia and remained increased throughout the entire period of hyperthermia. Sperm motility, count, and viability were significantly decreased compared with the respective controls as early as D20, D34 and D34 respectively during hyperthermia and remained decreased during the entire hyperthermia period. After cessation of hyperthermia all parameters returned to respective control values at D73.

We report, for the first time in men, that a mild increase in testes and epididymal temperature largely impaired sperm chromatin integrity before any drop in sperm count. Based on the results from animal models the findings of present experimental protocol raise the question of possible consequences of altered sperm chromatin integrity on embryo development when sperm parameters were compatible with natural fertilization during male contraception particularly during inhibition and recovery phases of spermatogenesis. Moreover, our results may have clinical implications in male infertility, repeated miscarriages as well as assisted reproductive technologies.

Key words: sperm, DNA fragmentation, hyperthermia, SCSA, sperm count, epididymis, testicular temperature, male contraception, sperm chromatin.

ABBREVIATIONS

GPC	Glycerophosphochiline
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
FSH	Follicle stimulating hormone
BTB	Blood testes barrier
IVF	In vitro fertilization
IGF-1	Insuline like growth factor 1
INSL3	Insuline-like factor 3
TGFβ	Transforming growth factor beta
NGF	Nerve growth factor
GnRH	Gonadotropin releasing hormone
LH	Luteinizing hormone
DFI	DNA fragmentation index
SCSA	Sperm chromatin structure assay
TUNEL	Terminal deoxnucleotidyl transeferase dUTP nick end labelling assay
Hsp	Heat shock protein
COMET	Single cell gel electrophoresis test
hCG	Human chorionic gonadotropin
NADPH	Nicotinamide adenine dinucleotide phosphate
ICSI	Intracytoplasmic sperm injection
ART	Assisted reproductive techniques
ROS	Reactive oxygen species
DAC	Diurnal artificial cryptorchidism

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
HDS	High DNA stainability
DPBS	Dulbecco's phosphate buffer saline
PFA	Paraformaldehyde
NaOH	Sodium hydroxide
MAI	Multiple anomalies index
WHO	World health organization
AAB	Acid aniline blue test
FISH	Fluorescence in situ hybridization
PSSC	Premature separation of sister chromatids
PEB	Cisplatin, etoposide and bleomycin
SSC	Saline sodium citrate
NP40	Nonyl phenoxypolyethoxylethanol

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INTRODUCTION

Reproduction is a biological process by which new offsprings are produced from their parents. It is a fundamental feature of every known life and is opposite to death. The known methods of reproduction are grouped as: asexual and sexual reproduction. In asexual reproduction, an individual can reproduce without the involvement of another individual of that species like in plants and bacteria. Sexual reproduction requires the equal involvement of the two individuals or gametes, one of each from opposite sex. To produce a healthy offspring, the quality and genetic integrity of both gametes is equally important. When the quality of any of the gamete is compromised disturbances in fertility emerge. Fertility refers to the capability to conceive or induce a pregnancy. For example, when a couple fails to conceive during one year of unprotected sex the term infertility is used. Both partners, male and female, contribute significantly in infertility disturbances; however, the unknown causes of infertility are not negligible too.

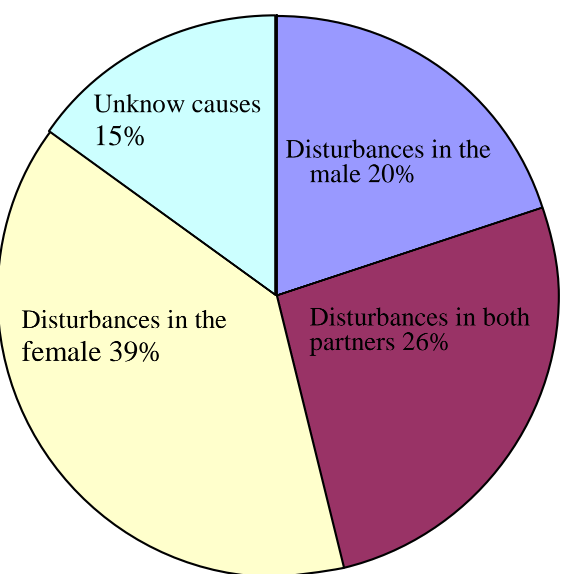

Figure 1. Distribution of causes of involuntary childlessness between men and women (Modified from Nieschlag *et al.,* 2010).

Male factor disturbances are important when infertility is taken into account. Half of the genetic material is contributed by male gamete, therefore, any deformity or abnormality in male gamete may lead to infertility and depending upon the conditions, complete absence of spermatozoa may lead to male sterility. Among the different causes of male infertility high testicular temperature attains an important consideration. The reason is that in man and most of the mammals, testes are anatomically placed in a position where temperature is lower than body temperature, therefore, testes functioning is temperature sensitive. Any variation in testicular temperature, high or low, may change the out put of testicular performance. Human testes encounter several conditions in daily life where temperature increases. If we look at it we find an increase in the testicular temperature during drive, sleeping, sitting for longer periods, sitting postures, taking hot baths, wearing tight underwear etc. Taking into consideration certain pathological conditions like varicocele and cryptorchidism, we also find a rise in testicular temperature resulting into spermatogenesis impairment. Though, reasonable data are available on animal models expressing the deleterious effects of higher testicular temperature on spermatogenesis but majority of these experiments have been performed above physiological temperature. Further, a human model is lacking which can give a detailed picture of the temporal effects of testicular hyperthermia on spermatogenesis. In continuation to this quest, the main objective of this research work was to investigate the effects of mild increase of testicular and epididymal temperature on kinetics of spermatogenesis, sperm parameters and chromatin/DNA integrity. The important aspect of these experiments is that the testicular temperature was maintained equal or slightly less than physiological temperature of human body.

In the next section, a brief overview of physiology of male reproductive tract, testes and epididymis is described.

1. Physiology of male reproduction

1.1. Overview

1.1.1. Human male reproductive tract

In man, reproductive tract is comprised of, two testicles, two epididymides, extensions of epididymis at both sides called vas deference, accessory sex glands known as; seminal vesicles, prostate glande, bulbouretheral glands or cowper's gland, a penis and a scrotum. The different parts of male reproductive tract play an essential role in male reproduction. The testes are the key organ in male reproduction responsible for sperm production.

1.1.1.1. Anatomical and functional organisation of testes

Testes are glandular organs suspended by spermatic cord in a cutaneous pouch like structure called scrotum. During early fetal life they are contained in the abdominal cavity but latter on before birth they descend into the scrotum.

The parenchyma of the testes is comprised of two compartments:

a) interstitial compartment

b) tubular compartment

1.1.1.1.1.. Interstitial compartment

The most important cells of the interstitial compartment are the leydig cells. Leydig cells are responsible for the production of testosterone and Insulin-like factor 3 (INSL3). This compartment also contains immune cells, blood cells, lymph vessels, nerves and fibroblasts and loose connective tissue. In human testis the interstitial compartment represents 12-15

% of the total testicular volume, 10-20% of which is occupied by leydig cells. It is estimated that human testes contain approximately 200 million leydig cells.

1.1.1.1.2. Tubular compartment

This compartment contains germ cells and two types of somatic cells; the Sertoli cells and the peritubular cells. The compartment is divided into about 250-300 lobules by septa of connective tissue. Each lobule contains 1-3 highly convoluted tubules called seminiferous tubules. It is thought that a human testis contains about 600 seminiferous tubules. The average length of each seminiferous tubule is about 60 cm (range 30-80 cm), therefore, the total length of all tubuli seminiferi per man is 720 m (360 m per testis). On average this compartment represents about 60-80% of the total testicular volume.

1.1.1.2. Anatomical and functional organisation of epididymis

Epididymis is a tubular organ which receives spermatozoa from testis. They are two in number, one on each side of each testis. The epididymis can be divided into three parts according to the functional commitments of the organ:

a) *caput/head*
b) *corpus/body*
c) *cauda/tail*

Principal functions of epididymis are the absorption and secretion of fluids which are essential for the maturation of spermatozoa. Here spermatozoa are matured and stored prior to ejaculation. It has a pseudostratified epithelium which contains principal cells of various heights, basal cells and lymphocytes. The fixed cells are both resorptive and secretory in nature and sperm can survive for 2 weeks in this environment. Depending

upon the testicular sperm output it takes on average 12 days for spermatozoa to pass through epididymis. Three low molecular weight secretions are present in high concentration in epididymis. These are: L-carnitine, myo-inositol and glycerophosphocholine (GPC).

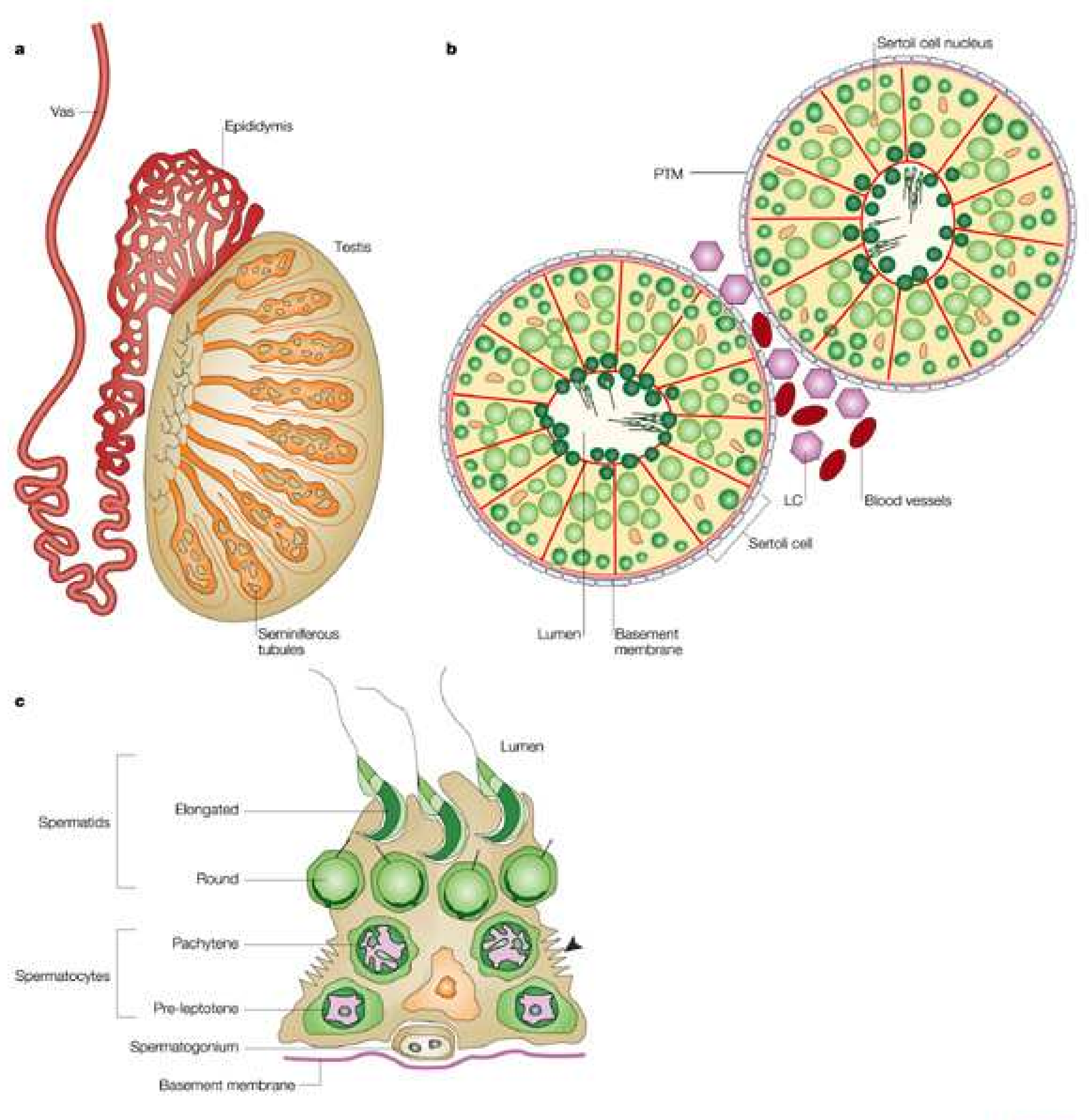

Figure 2. (a) Cross-section through a testis, showing the location of the seminiferous tubules, the vas deferens and the epididymis. (**b)** A diagrammatic cross-section through a

testicular tubule, showing the germ cells (green) at different stages of maturation. (**c**) A single Sertoli cell with its associated germ cells (Cooke and Saunders, 2002).

1.1.1.3. Male reproductive accessory glands

The accessory glands of the male reproductive tract are the seminal vesicles, prostate and the bulbourethral glands (Fig 3).

1.1.1.3.1. Seminal vesicles

These are the paired glands in the posterior of the urinary bladder. Each gland has a short duct that joins with the ductus deferens at the ampulla to form the ejaculatory duct which then empties into the urethra. The fluid of these glands is viscous and contains fructose that provides energy to the spermatozoa; prostaglandins, which contribute to the mobility and viability of sperm, and proteins that cause slight coagulation reactions in the semen after ejaculation.

1.1.1.3.2. Prostate

The prostate gland is a firm, dense structure that is located just inferior to the urinary bladder. It is about the size of a walnut and encircles the urethra as it leaves the urinary bladder. Numerous short ducts from the substance of the prostate glands empty into the prostatic urethra. The secretions of the prostate are thin, milky colour and alkaline. They function to enhance the motility of the sperm. Enzymes of prostate gland are responsible for the liquefaction of the semen after ejaculation. Zinc and citric acid are the major markers present in the secretions of prostate gland. Zinc helps to stabilize the sperm chromatin and its deficiency may result into lowered fertility because it may lead to high sperm fragility (Canale *et al.*, 1986).

1.1.1.3.3. Bulbourethral glands

The paired bulbourethral (Cowper's) glands are small, about the size of a pea, and located near the base of the penis. A short duct from each gland enters the proximal end of the penile urethra. In response to sexual stimulation, the bulbourethral glands secrete an alkaline mucus-like fluid. This fluid neutralizes the acidity of the urine residue in the urethra, helps to neutralize the acidity of the vagina, and provides some lubrication for the tip of the penis during intercourse.

Semen

Semen (seminal plasma + sperm cells) is a slightly alkaline mixture of sperm cells and secretions from the accessory glands. Secretions from the seminal vesicles make up about 60-65 percent of the volume of the semen, with most of the remainder coming from the prostate gland. The secretions from the bulbourethral gland contribute only a small volume.

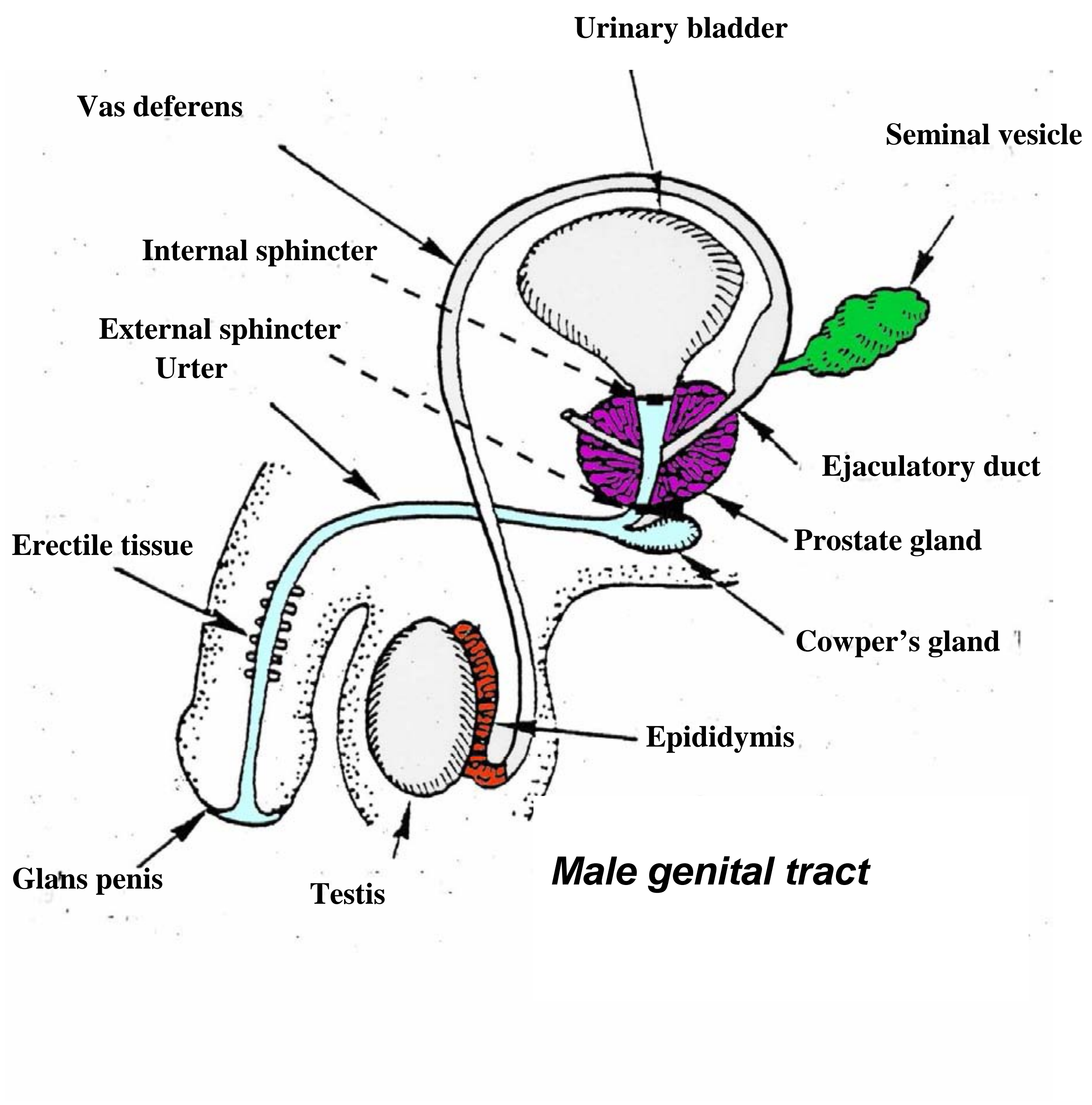

Figure 3. Organs of male reproductive tract (Modified from "lectures on medicine of reproduction" by Pr.Bujan Louis).

The next section deals with the role of testes in spermatogenesis and maturation of spermatozoa in epididymis.

1.1.2. Spermatogenesis

Spermatogenesis takes place in seminiferous tubules in the testes. It starts with the division of germ cells arranged in particular cellular associations known as spermatogenic stages and ends with the formation of spermatozoa. The whole spermatogenic process can be divided into four phases:

1) *Mitotic phase:* in this phase the diploid spermatogonia (germ cells) proliferate and differentiate mitotically. This phase can also be named spermatogoniogenesis.
2) *Meiotic phase:* diploid spermatocytes divide and produce haploid germ cells called spermatids.
3) *Spermiogenesis:* transformation of spermatids into testicular sperm.
4) *Spermiation:* in this phase the elongated spermatids from germinal epithelium are released into the tubular lumens which later become spermatozoa.

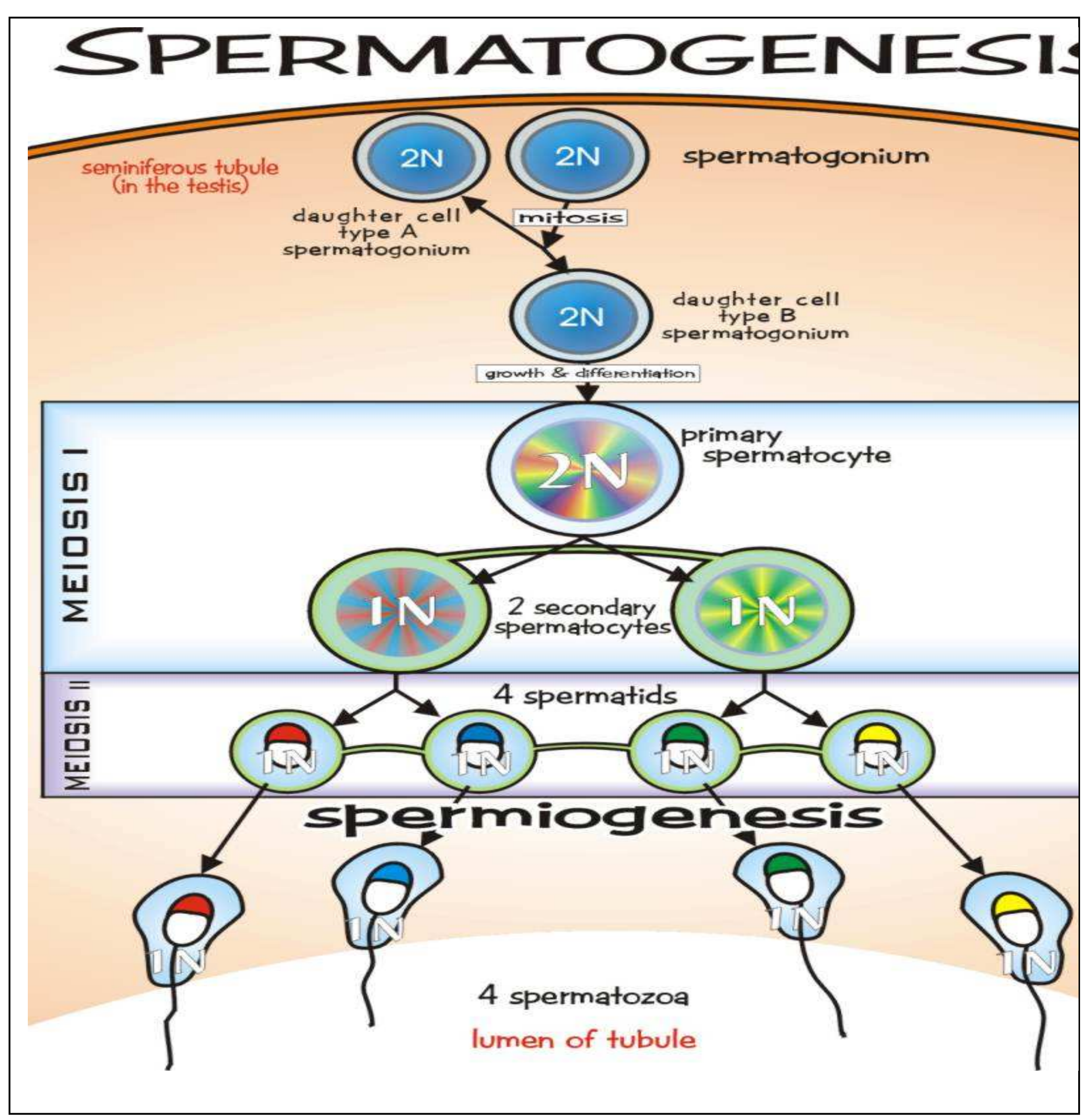

Figure 4. Germ cell division, proliferation and differentiation during spermatogenesis (http://www.tokresource.org/tok_classes/biobiobio/biomenu/reproduction/spermatogenesis.jpg).

1.1.2.1. Spermatogoniogenesis

Spermatogonia lying at the basal part of the germinal epithelium can be distinguished by their position, their morphology and stainability of nuclei. Broadly, they are classified into two types: A type and B type spermatogonia. Type A spermatogonia are further classified into Ad (dark) and Ap (pale) spermatogonia. It is believed that Ad spermatogonia do not divide but rarely (Ehmcke and Schlatt, 2006). These spermatogonia are considered to represent the testicular stem cells (Ehmcke *et al.*, 2006). However, these spermatogonia undergo mitosis when the reserve of testicular stem cells is in danger like in case of radiation (de Rooij, 1998). On the other hand, Ap spermatogonia divide into two daughter B spermatogonia as well as Ap spermatogonia where Ap spermatogonia serve as reservoir. B spermatogonia then divide to form preleptotene spermatocytes or primary spermatocytes. Spermatogonia multiply continuously in successive mitosis but these cell divisions are usually incomplete. The daughter cells remain interconnected by cytoplasmic bridges and a syncytium of cells is formed originating from a clone of stem cells. In cases of reduced spermatogenesis A dark-type spermatogonia are usually absent and in the absence of both types of spermatogonia no spermatogenesis takes place and the germinal epithelium consists of Sertoli cells only. There may be congenital absence of spermatogonia (Sertoli cell-only Syndrome) or Sertoli cells may be destroyed by other factors such as radiations, chemotherapy etc (acquired Sertoli cell-only Syndrome).

1.1.2.2. Meiotic phase

The stage of meiosis undergoes changes in the nuclear chromatin configuration after the last spermatogonial division. This stage is comprised of two divisions, meiosis I and meiosis II. Cells before the fist division are called primary spermatocytes (spermatocytes I)

and cells before second division are called secondary spermatocytes (spermatocytes II). Leptotene stage of first prophase (meiosis I) takes places in the basal compartment of the germinal epithelium then spermatocytes pass the Sertoli cells barrier and reach the adluminal compartment. In adluminal compartment further stages of meiosis namely, zygotene, pachytene and diplotene occur. During S phase DNA reduplicates and chromosomes condensations occure during prophase. In metaphase, homologous chromosomes form pairs and crossing over takes place in the later steps. Primary spermatocytes are the largest germ cells and after first meiotic division give rise to secondary spermatocytes which contain a haploid set of chromosome but in duplicate form. Secondary spermatocytes then undergo second meiotic division and form haploid cells called spermatids. The prophase of first meiotic division lasts 3 weeks and the other phases of first meiotic division and whole of the second meiotic divisions take 1-2 days. During meiosis genetic recombination and exchange of genetic information takes place which is essential for the diversity of life. At this stage of spermatogenesis genetic modifications can occur due to disjunction or wrong pairing of homologous chromosomes. Some times large spermatocytes (megalo- spermatocytes) appear in the ejaculates due to asynapsis of homologous chromosomes.

1.1.2.3. Spermiogenesis

Haploid cells formed after second meiotic divison are called spermatids. These are mitotically inactive but they undergo differentiation and produce elongated spermatids and sperm. These processes include condensation and shaping of the cell nucleus. Here the flagellum is formed and a large part of cytoplasm is extruded. Spermiogenesis can be divided into four phases (Nieschlag *et al.,* 2010):

a) Golgi phase: acrosomal bubbles and craniocaudal symmetry appear.

b) Cap phase: spermatids become elongated and acrosome develops.

c) Acrosomal phase: during this phase the cell nucleus is further condensed and cell elongation continues.

d) Maturation phase: in this phase the extrusion of the rest of the cytoplasm occurs.

During the process of spermiogenesis remodelling of sperm chromatin occurs. Chromatin remodelling requires well planned and regulated post-translational modifications of histones which also includes acetylation (Meistrich *et al.*, 1992; Marcon and Boissonneault, 2004), ubiquitination (Lu *et al.,* 2010; Chen *et al.*, 1998; Baarends *et al.*, 1999), methylation (Godmann *et al.*, 2007) and phosphorylation (Leduc *et al.*, 2008). After the hyperacetylation of histones nucleosomes are destabilized to become prepared for the replacement of transition proteins with protamines (Pivot-Pajot *et al.*, 2003; Kurtz *et al.*, 2007). Alterations in the sperm chromatin remodelling during spermiogenesis could result into DNA fragmentation. The presence of strand breaks (nicks) in the ejaculated spermatozoa may indicate the incomplete maturation during this process (McPherson and Longo, 1993a; McPherson and Longo, 1993b; Marcon and Boissonneault, 2004). Abnormalities in the controlling mechanism of spermiogenesis could result in anomalies of chromatin packaging and DNA strand breaks which make the sperm more susceptible to post testicular assaults.

As a result of this stage a large number of spermatids are formed. Here malformations usually affect acrosome, nucleus and flagellum and some times may be combined defects resulting into a large number of malformed spematids (round headed, multinucleated spermatids, disturbances in nuclear condensation, flagellum malformations i.e. absent or multiple flagella) (Holstein *et al.*, 2003).

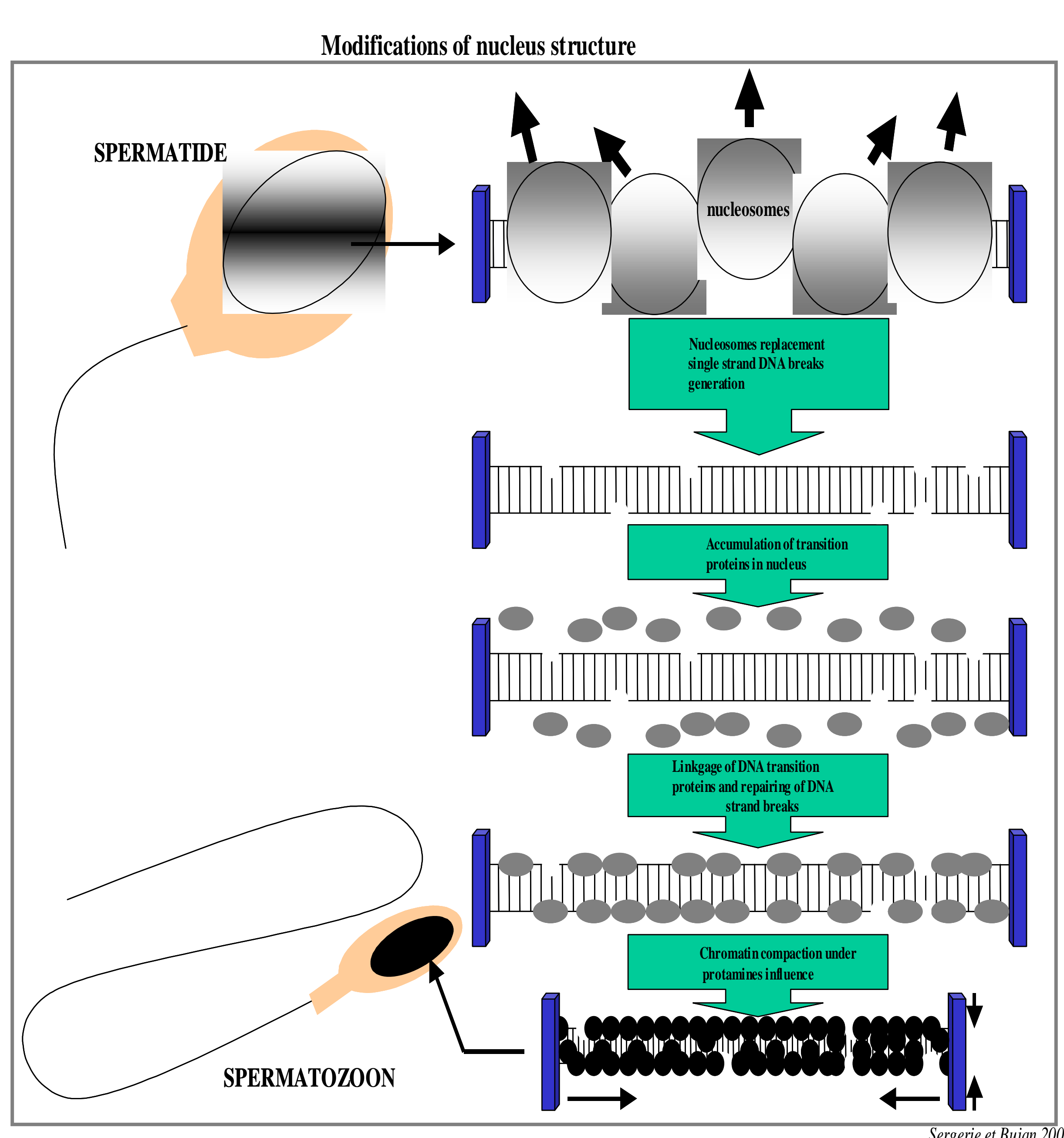

Figure 5. Nuclear compaction during spermiogenesis (Modified from Sergerie *et al.*, 2005).

1.1.2.4. Spermiation

In this phase the spermatids are released into the lumen of the seminiferous tubules. This process can be particularly affected by hormonal modifications, temperature and toxins. Sertoli cells play a role in the movement of spermatids to the border of the lumen of seminiferous tubules. The intercellular bridges are disconnected and spermatids become free cells now called spermatozoa. Residual bodies are formed comprising of smaller parts of spermatids with cumulated RNA granules, mitochondria and lipid droplets which are digested by Sertoli cells (Breucker *et al.*, 1985).

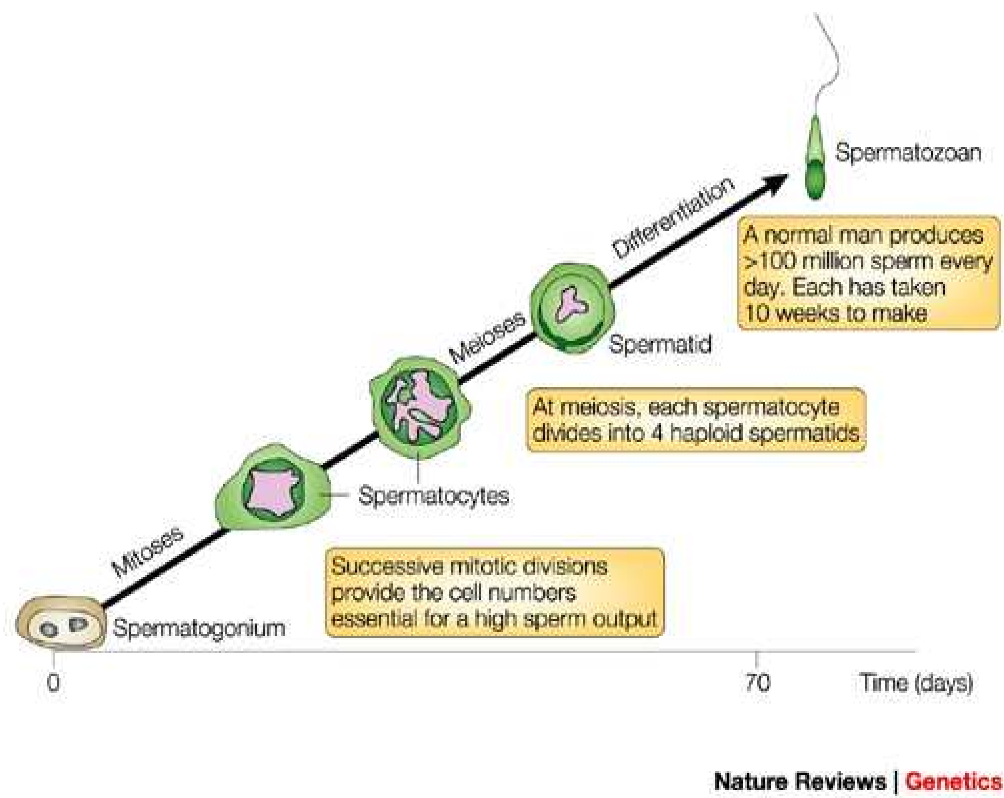

Figure 6. Stages of spermatogenesis: time course of human spermatogenesis (Cooke and Saunders, 2002).

Cycle of seminiferous epithelium in human

It is believed that in adult, germ cell development follows a specific timescale, with minor biological variations. In man it takes 16 days for spermatogonia to become committed to enter spermatogenesis process. Because duration of spermatogenesis is very long (74 day in man, Fig 7) compared to the interval between commitments of spermatogonia (16 days), cohort of germ cells is layered, with the youngest near the basement membrane. These layers are sometimes termed generations. Collectively in human 6 cohorts of germ cells are found in a circumscribed area of seminiferous tubules called cellular associations. Collectively, from multiple points within the seminiferous tubules of normal human testis, more than 25 000 sperm are released each and every minute (Amann, 2008).

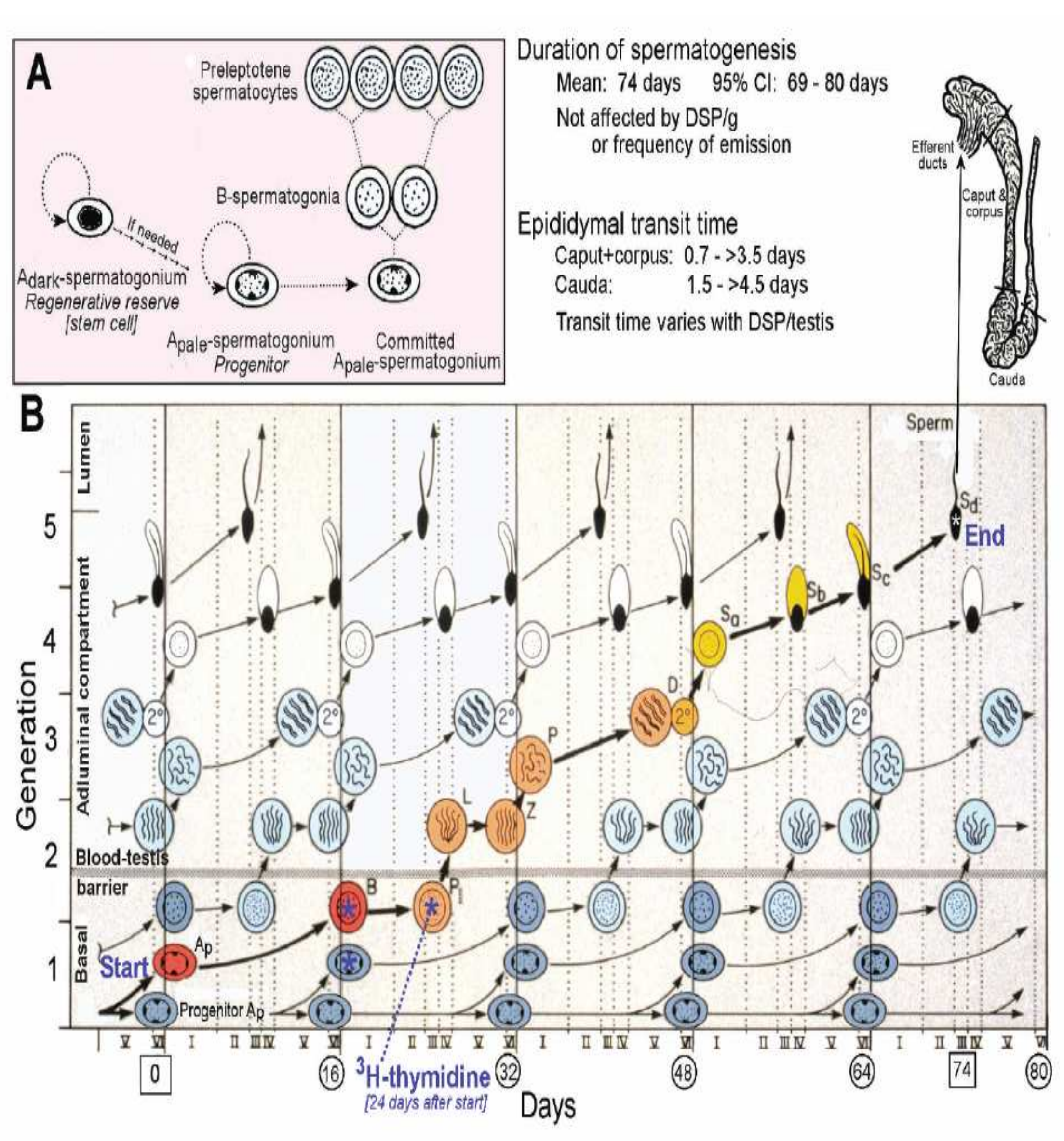

Figure 7. Spermatogenic cycle in man (Amann, 2008).

1.1.3. Role of Sertoli cells in spermatogenesis

Sertoli cells synthesize and secrete several factors: proteins, cytokines, growth factors, opioids, steroids, prostaglandins, modulators of cell division etc. The morphology of Sertoli cells is strictly related to their physiological functions. They secrete inhibin and activin after puberty which work together to regulate FSH secretions. Estradiol-aromatase from Sertoli cells converts testosterone to 17 beta estradiol to direct spermatogenesis. They participate in formation of the blood testes barrier (BTB) which prevents the mixing of toxins and other harmful agents in the blood of interstitial compartment with the adluminal compartment of seminiferous tubules (Fig 8). These cells help in renewal of the germ cells and then support their stepwise development and differentiation into mature spermatozoa. During maturation of spermatozoa they also engulf the unnecessary portions of spermatozoa.

There is a specific number of Sertoli cells depending upon the species. In man 10 germ cells or 1.5 spermatozoa per Sertoli cells are observed (Zhengwei *et al.*, 1998). About 35-40% of the volume of germinal epithelium is represented by Sertoli cells. The intatct testis with complete spermatogenesis contains 800-1200 million Sertoli cells (Zhengwei *et al.*, 1998) or approximately 25 million Sertoli cells per gram of testis (Raleigh *et al.*, 2004).

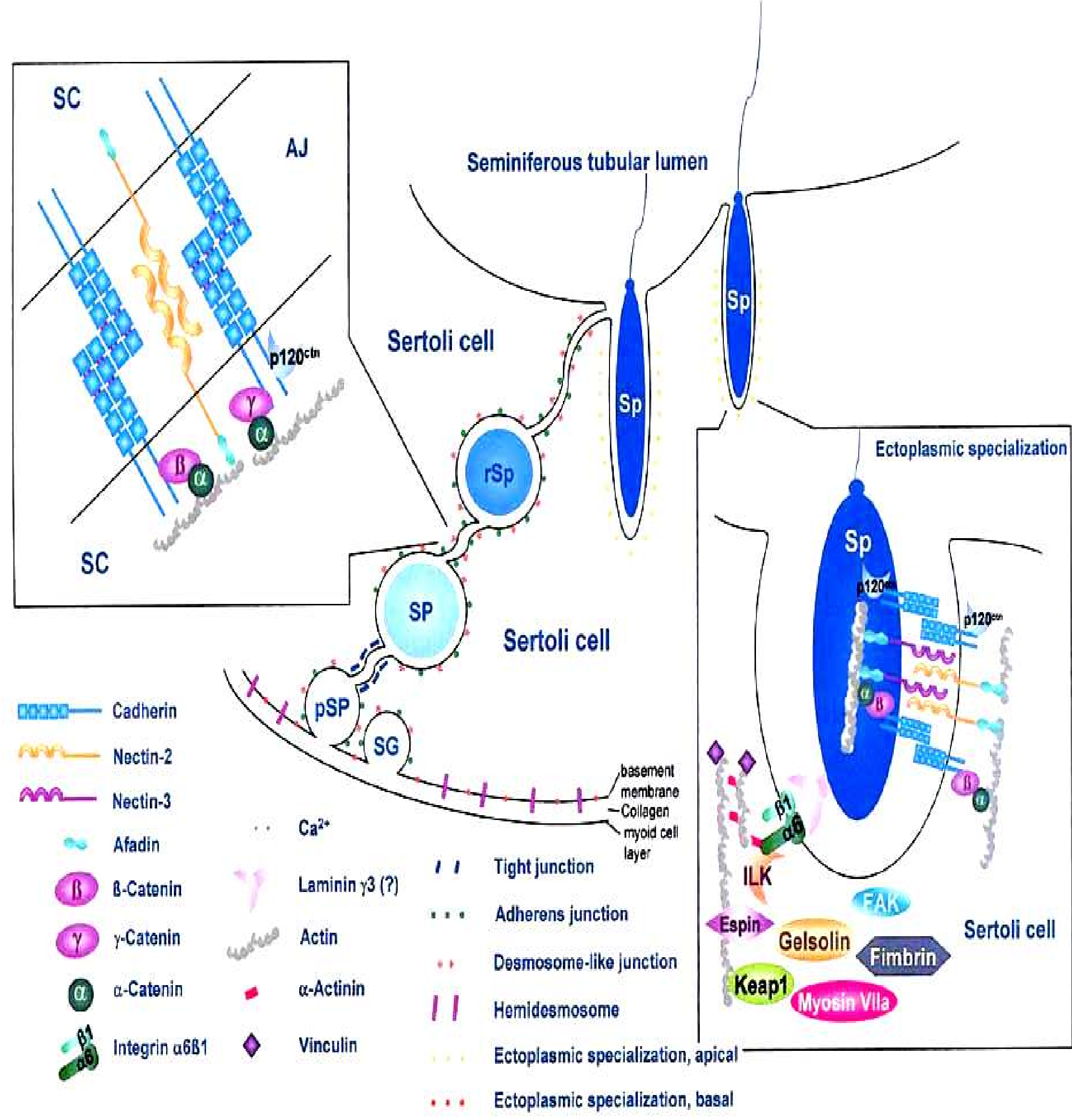

Figure 8. Schematic diagram illustrating the structural units of the adherens junctions in testicular germinal epithelium (Lui *et al.,* 2003).

1.1.4. Role of epididymis in sperm maturation

Sperm maturation is a process occurring in the caput and corpus of the epididymis where spermatozoa gain their fertilizing ability. The process is associated with many physiological and morphological changes in the spermatozoa (Cooper, 2007). Spermatozoa while passing through the epididymis acquire changes in morphology, sperm motility and specific motion pattern (Yeung *et al.*, 1993; Soler *et al.*, 2000). Epididymis plays an important role in acquiring the sperm zona binding capacity in distal parts of epididymis (Moore *et al.*, 1992). The chromatin of sperm becomes more condensed during epididymal transit (Haidl *et al.*, 1994; Golan *et al.*, 1996). Also when distal epididymal spermatozoa are used in IVF cycles, higher rates of fertilization and pregnancy have been achieved (Patrizio *et al.*, 1994).

Due to the anatomical positioning of the testes in man, the degree of increase or decrease in testicular temperature may modify the spermatogenesis. Before citing the literature on the effects of thermal variations on sperm production it will be interesting to explain the thermoregulation mechanisms of testes.

1.1.5. Testicular blood supply

1.1.5.1. Testicular artery

It originates from the aorta, runs obliquely through the abdominal wall via the inguinal canal. Then it becomes flexuous from the deep inguinal ring. In all eutherian mammals with scrotal testes, the artery is unique, except for the branches to the epididymis, two in man, superior to the head and inferior to the body and tail (Harrison and Barclay, 1948)

1.1.5.2. Testicular veins

In man venous circulation of testes includes two major drainage networks:

1) Deep venous system: drains the testes, epididymis and deferent ducts and,
2) Superficial venous network drains the scrotum (Bensussan and Huguet, 1984)

Deep venous network comprises of:

a) Pampiniforme plexus

b) Posterior spermatic plexus

c) Veins of deferent ducts

Superficial venous network comprises of:

a) Superficial veins of scrotum

b) Deep veins of scrotum

1.1.6. Regulation of spermatogenesis

The process of spermatogenesis is influenced by different internal and external factors. Broadly two types of regulations are cited in literature:

a) Intrinsic regulation

b) Extrinsic regulation

1.1.6.1. Intrinsic or auto/paracrine regulation

The Leydig cells in the intertubular space secrete testosterone and additional neuroendocrine substances and growth factors. These hormones, transmitters and growth factors are directed to neighbouring Leydig cells, to blood vessels, to the lamina propria of the seminiferous tubules and to Sertoli cells (Fig 9). They are involved in maintenance of the trophic of Sertoli cells and the cells of the peritubular tissue; they influence the

contractility of myofibroblasts and in that way regulate the peristaltic movements of seminiferous tubules and the transport of spermatozoa (Middendorff *et al.*, 1997). They also contribute to the regulation of blood flow in the intertubular microvasculature. Furthermore, different growth factors (IGF1, TGFβ, and NGF) are delivered from Sertoli cells and several types of cytokines take part in a complicated circle of regulation of cell functions and developmental processes of germ cells. Taken together all these factors represent an independent intratesticular regulation of spermatogenesis. This very intricate system has been investigated mainly in animals and is not as well understood in human (Holstein *et al.*, 2003).

1.1.6.2. Extrinsic or endocrine regulation

Local regulation of spermatogenesis in the testis requires the well known extratesticular stimuli provided by the hypothalamus and hypophysis. Pulsatile secretion of gonadotropin releasing hormone (GnRH) of the hypothalamus initiates the release of luteinizing hormone (LH) of the hypophysis. As a result of this stimulus Leydig cells produce testosterone. Testosterone influences not only spermatogenesis in the seminiferous tubules of the testis but is also distributed throughout the body and provides feedback (after aromatization in estradiol) to the hypophysis and hypothalamus related to the secretory activity of Leydig cells. Stimulation of Sertoli cells by the pituitary follicle stimulating hormone (FSH) is necessary for the maturation of germ cells. The Sertoli cells themselves secrete inhibin in the feedback mechanism directed to the hypophysis. The extratesticular influences are a necessary basis for the function of intratesticular regulations (Fig 9) (Holstein *et al.*, 2003).

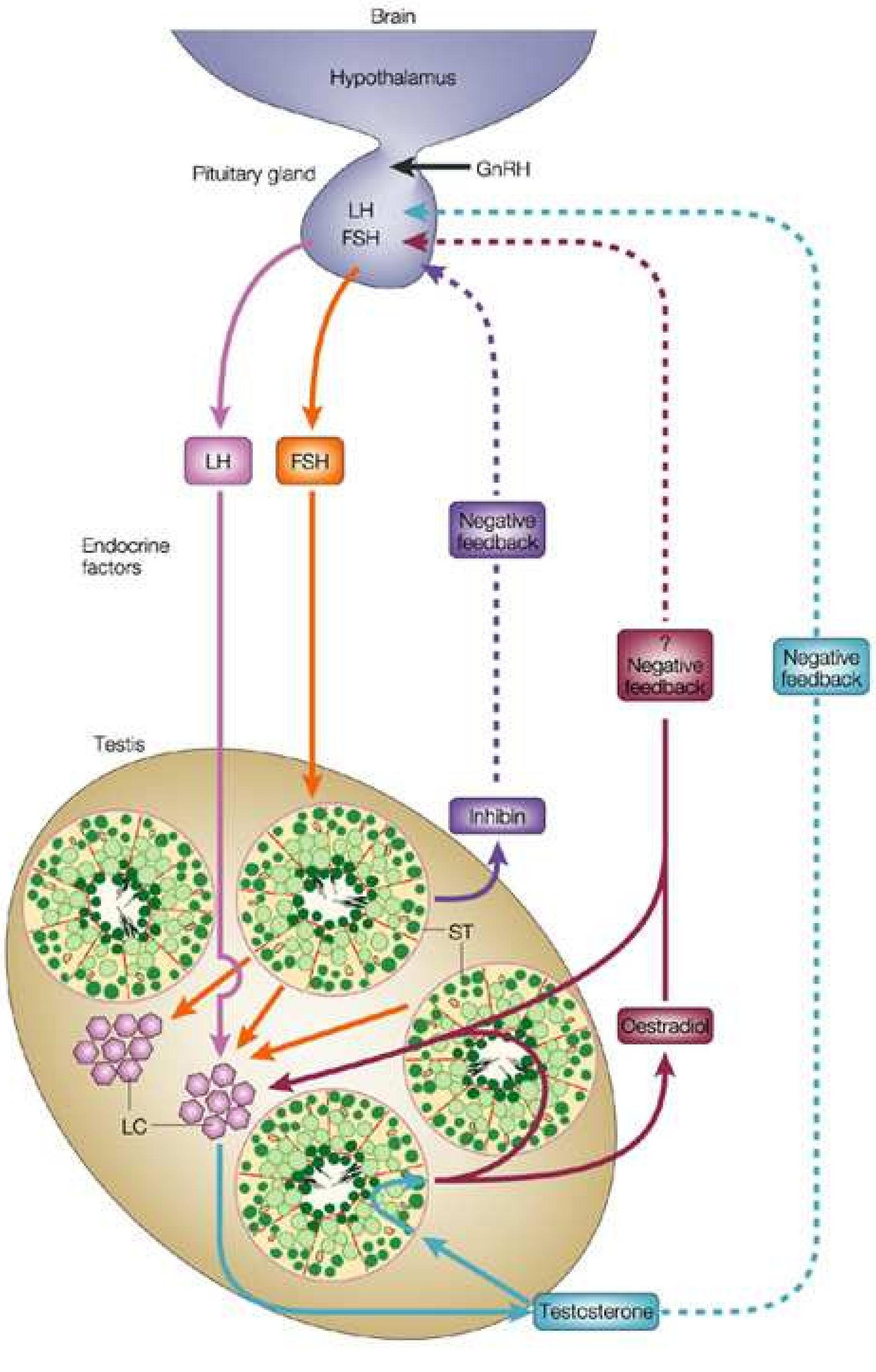

Figure 9. Intrinsic and extrinsic regulations of spermatogenesis (Cooke and Saunders, 2002).

1.1.6.3. Thermoregulation of spermatogenesis

In man testes are at a temperature 2-3 °C lower than the body temperature. This decrease in the testicular temperature is necessary for the production of fertile spermatozoa. Any change in the testicular temperature has the potential to change the fate of spermatogenesis. Therefore, temperature regulation in testicles is of prime importance for normal fertility. This temperature regulation is carried out by different structural components of which pampiniform plexus is of most importance. Secondly, scrotum in man has a special role in temperature regulation of the testes.

1.1.6.3.1. Pampinifrom plexus

This is a network formed, in the male, by veins from the testicle and epididymis, consisting of eight or ten veins lying in front of the ductus deferens and forming part of the spermatic cord. The spermatic veins emerge from the back of the testis, and receive tributaries from the epididymis: they unite and form the pampinifrom plexus which forms the chief mass of the cord. The vessels composing this plexus are very numerous, and ascend along the cord in front of the ductus deferens; below the subcutaneous inguinal ring they unite to form three or four veins, which pass along the inguinal canal, and, entering the abdomen through the abdominal inguinal ring, coalesce to form two veins (Fig 10).

1.1.6.3.2. Scrotum

Scrotum is involved in local control of temperature by three different but overlapping mechanisms, which are changes in blood flow, adaptability of the scrotal surface area and finally the evaporation of sweat.

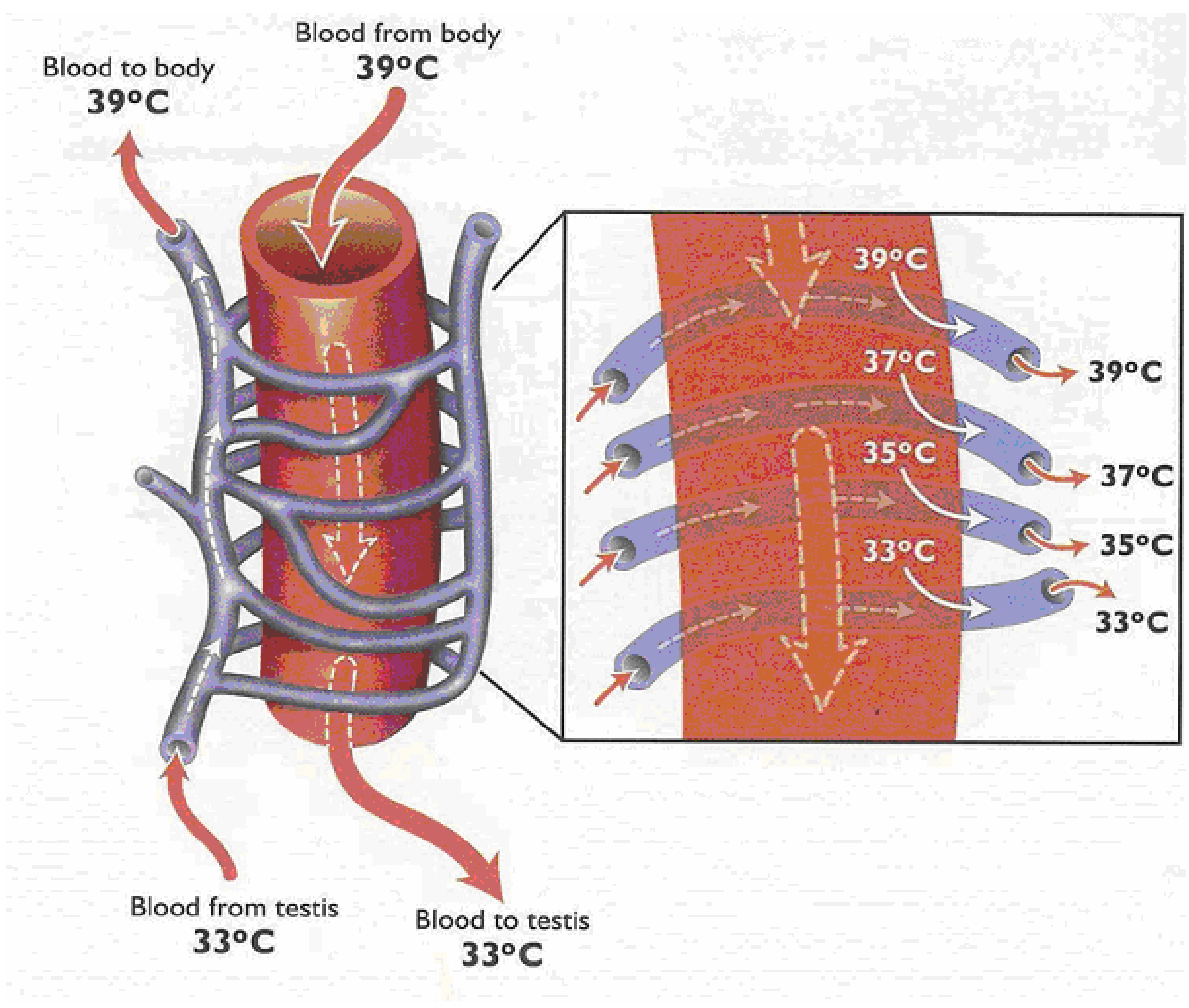

Figure 10. Counter current mechanism of heat exchange a model of ram (Adopted from "lectures on clinical andrology" by Dr Mieusset Roger).

Scrotal blood flow

When an elevation in scrotal temperature was induced in anesthetized rats (Waites *et al.*, 1973) or rams (air) (Fowler, 1968; Fowler and Setchell, 1971), the scrotal blood flow was increased. This increase in blood flow resulted from a mild increase in scrotal temperature which appeared as early as less than 20 minutes in rats and it became very intense (multiplied by two) when scrotal temperature passed from 33 to 37 °C in rams or rats. Furthermore, a temperature increase up to 40, 43 and 45 °C caused an increase in scrotal blood flow. However, at these higher temperatures this increase in scrotal blood flow does not appear uniformly in all regions of scrotum but mainly in posterior region (rams) (Fowler, 1968).

Variability in scrotal surface area

In rams, the degree of the dartos muscle contractions is constantly adapted to values close to normal testicular temperature in response to changes in skin temperature. Scrotum starts relaxing when scrotal skin temperature is 34 °C. When the dartos is completely relaxed the scrotal air temperature is 20% superior to its normal values, which allows exchange of fluids and evaporation (Phillips and McKenzie, 1934).

Evaporation

In men, unlike rodents, the scrotum is devoid of sweat glands, the fluid exchange occurs by simple diffusion (Buettner, 1969). Secondly, evaporation of liquids brought to the surface of the scrotal skin, sweating, or by simple diffusion causes further heat loss establishing the role of scrotum in thermoregulation (Buettner, 1969).

1.2. LITERATURE REVIEW

2.1. Temperature and male fertility

2.1.1. Testicular temperature and sperm parameters in animals

Normal functioning of the mammalian testis is ensured at a temperature 2-3 °C cooler than body temperature. Over the last century, the link between high testicular temperature and male fertility has been addressed by many scientist and clinicians. Earlier animal reports on the temperature associated testicular performance were delivered by Fukui (1923), Moore and Chase (1923) and Young (1927), showing testicular degeneration and sperm parameters alterations at a temperature higher than normal testicular temperature. Afterwards, the effects of testicular and scrotal hyperthermia and alterations in sperm parameters were reported in different animal species *i.e.* in rams (Dutt and Simpson, 1957; Fowler and Dun, 1966; Fowler, 1968; Rathore, 1968, 1970; Howarth, 1969; Mieusse*t et al.,* 1992a), mice (Burfening *et al.,* 1970; Bellvé, 1972, 1973; Meistrich *et al.,* 1973; Setchell *et al.,* 1998; Zhu *et al.,* 2004; Paul *et al.,* 2008b; Pérez-Crespo *et al.,* 2008), rats (Chowdhury and Steinberger, 1964) and rabbits (Howarth *et al.,* 1965; Burfening and Ulberg, 1968). These studies have reported alterations in sperm characteristics after testicular/scrotal or whole body hyperthermia.

2.1.2. Testicular temperature and sperm parameters in men

After preliminary animal studies, researchers undertook the investigations in man. A series of studies was then carried out interrogating the adverse effects of induced hyperthermia on human spermatogenesis such as scrotal hyperthermia (Watanabe, 1959; Robinson and Rock, 1967; Rock and Robinson 1965; Robinson *et al.,* 1968), testicular hyperthermia (Mieusset *et al.*, 1985; Mieusset *et al.*, 1987a; Mieusset *et al.*, 1987c) or whole body

temperature rise (Procope, 1965). Attempts have also been made to address a male contraception method after elevating the testes through the inguinal canal thus rising the temperature near to body temperature for different periods of time in healthy men. These experiments allowed the authors to demonstrate not only drastic quantitative (Mieusset *et al.*, 1985) but also qualitative (Mieusset *et al.*, 1987c) alterations in spermatogenesis. These findings led the researchers to highlight the efficacy of this contraception method which was reversible (Mieusset and Bujan, 1994). Further, they carried out clinical trials of various suspensory designs as male contraceptives. Their results showed that men who wore one suspensory design with a rubber ring to hold the testes in the inguinal canals had ~100% effective contraception. All trial participants achieved very low motile sperm counts, between 0 and 1.6 million sperm per millilitre during experimentation (Mieusset and Bujan, 1994).

In continuation to the work on male contraception, another series of studies (Shafik, 1991a, b) described a reversible and safe male contraception method by testicular suspension. In 28 male volunteers the testes were suspended in the superficial inguinal pouch close to the scrotal neck using 2 approaches like; stitch and ball suspension for 12 months. During suspension sperm count dropped to severe oligospermia and pregnancy could not occur. However, 6 months after suspension release sperm count returned to normal, testicular biopsy showed normal spermatogenesis and pregnancy occurred (Shafik, 1991a). Further, in 1992, working on 14 men Shafik demonstrated another contraceptive technique by the use of a polyester sling applied on the scrotum. After 12 months of polyester sling men were rendered azoospermic and recovery to normal sperm parameters occured 6 months after the suspension release (Shafik, 1992). The above cited data support the idea that testicular hyperthermia effectively suppresses spermatogenesis leading to male hypofertility.

2.1.3. Heat stress factors

2.13.1. Exogenous factors

Apart from the experimental testicular hyperthermia there are other conditions associated with testicular heat stress, such as, sitting and sleeping postures, occupation, car driving, tight clothing etc. (Bigelow *et al.*, 1998; Bujan *et al.*, 2000; Hjollund *et al.*, 2000; Hjollund *et al.*, 2002; Jung *et al.*, 2005; Koskelo *et al.*, 2005) previously described in a review (Thonneau *et al.*, 1997). In a more recent study, the authors (Mieusset *et al.*, 2007) studied the effects of posture and clothing on scrotal temperature in fertile men. This study elucidates a linkage between scrotal temperature and the posture. The above cited studies and the studies summarised in Table 1 of this section collectively suggest that daily life style, occupation and clothings alter testicular temperature and may lead to impaired spermatogenesis (Table 1).

2.1.3.2. Endogenous factors

Fever

Fever impairs spermatogenesis due to increased testicular temperature (Table 1) (French *et al.*, 1973; Sheriff, 1987; Evenson *et al.*, 2000; Sergerie *et al.*, 2007). Evenson *et al.*, (2000) by using SCSA in a fertile man presenting influenza and one day fever of 39.9 °C, reported 36%, 49% and 30% denatured DNA at 18, 33 and 39 days post fever respectively. The above study also reports alterations in the normal processing of protamine 2 and a slight increase in histone to protamine ratio suggesting a fever related disruption of the synthesis of mRNA which codes protamine 2 processing enzyme. A recent case report by Sergerie *et*

al., (2007) reported changes in sperm parameters (total sperm count, motility and vitality) and DNA fragmentation index (DFI) after febrile illness in a fertile man. Semen parameters were analysed before the episode of febrile illness and at days 15, 37, 58, 79 and > 180 after the fever. Total sperm count significantly decreased at 15, 37 and 58 after fever and returned back to normal by day 79 post fever. Percentage of motility significantly decreased by day 15 and 37 and returned to normal by day 58 post fever. DFI measured by sperm chromatin structure assay (SCSA) was significantly increased by 24% and 36% by days15 and 37 post fever respectively, and decreased to 15% and 8% approaching the days 58 and 79 post fever respectively.

Varicocele

Varicocele is another pathological condition which also increases the testicular temperature and may affect the sperm parameters (Table 1) but the results are contradictory (Goldstein and Eid, 1989; Lund and Nielsen, 1996; Jung *et al.*, 2002). Lund and Nielsen reported decreased sperm quality in men with varicocele but found no significant change in the mean testicular temperature of the testicles on the same side as the varicocele, 34.6 °C (±0.7) compared to the opposite side without varicocele 34.3°C (±0.6). However, they also reported that the mean core testicular temperature of both sides was lower in donors compared with men with varicocele and those exhibiting idiopathic oligozoospermia (Lund and Nielsen, 1996). On the other hand Jung *et al.,* (2002) found decreased semen quality as well as increased scrotal heat stress in patients with varicocele. Some more ancient studies also show discrepancy in results. For example, an elevation in testicular temperature was observed in men with varicocele (Hanley, 1956; Young, 1956) but a later study did not reveal any significant difference between the varicocele and control groups (Tessler and Krahn, 1966). Moreover, significant increase in scrotal temperature was observed in

infertile men (35±0.5 °C right, 35.1 ±0.6 °C left) having varicocele compared with controls (34.6±0.5 °C right and left) however, the difference was not significant when compared with infertile men without varicocele. Further, this study suggests the compromised sperm quality in case of higher scrotal temperature (Mieusset *et al.*, 1987c). A more recent study supports these findings that varicocele impairs the sperm quality with maximum effects in grade III varicocele (Mori *et al.*, 2008).

Cryptorchidism

Cryptorchidism alters sperm parameters by testicular hyperthermia and is the most frequent abnormality of male sexual differentiation. The study of Mieusset *et al.*, (1993) showed that temperature of the undescended testis, measured in its cryptorchid location during surgical procedure for orchidopexy in boys (n = 45), was significantly higher (34.4±0.9 °C) than that of the contralateral normally descended testicle (33.2±1.2 °C). Further, they reported that among the infertile men (N=1014), 95 men were with a history of cryptorchidism and 45% of these infertile men had abnormally higher scrotal temperature (Mieusset *et al.*, 1993). This abnormal scrotal temperature was proved to be a risk factor associated with impairement in spermatogenesis and a higher incidence of primary infertility compared with infertile men with history of cryptorchisdism but normal scrotal temperature (Mieusset *et al.*, 1995).

In short, heat stress associated exogenous and endogenous factors cause modifications in testes and epididymal temperature which in turn impair spermatogenesis as well as sperm chromatin integrity.

Unidentified causes

Interestingly, apart from the above discussed clinical conditions, Mieusset *et al., (2007)* reported a thermal difference between right and left scrotum in healthy volunteers. They found that the temperature of the right scrotum was higher than the left irrespective of the position of the man. They argued that this thermal difference between right and left scrotum could contribute to the asymmetry of male external genital organs (Mieusset *et al.*, 2007). They also reported an increased scrotal temperature in infertile men compared with fertile men without any identified factor (Mieusset *et al.*, 1989). The exact reason of such an increase in testicular/scrotal temperature is not known, one possible factor may be the impaired thermoregulation in such men.

Table 1. Synopsis of factors discussed to induce genital heat stress (Modified from Jung and Schuppe, 2007).

Authors	**Source of genital heat stress**	**Scrotal/testicular temperature**	**Semen quality/fertility parameters**
(Grove *et al.*, 2002)	Disposable plastic-lined diapers versus reusable cotton diapers	No temperature difference for the common use of cotton diapers with plastic pants	No data
(Hjollund *et al.*, 2000; Jung *et al.*, 2001; Jung *et al.*, 2003)	Genital insulation during sleep	Increased	Negative influence not documented
(Jung *et al.*, 2005; Koskelo *et al.*, 2005; Mieusset *et al.*, 2007)	Duration of sedentary posture	Increased	Negative influence not documented
(Hjollund *et al.*, 2002)	Increased mean daytime scrotal temperature	–	Reduced
(Thonneau *et al.*, 1997; Bujan *et al.*, 2000)	Professional drivers	Increased	Reduced, predominantly drivers of vans, trucks or industrial heavy machinery affected (confounders?)
(Sheynkin *et al.*, 2005)	Sitting with portable computers in a laptop position	Increased	No data
(Song and Seo, 2006)	Sitting on heated floors	Increased	No data
(Rock and Robinson, 1965)	Tight-fitting underwear	Increased	Negative influence not sufficiently proven
(Thonneau *et al.*, 1997; Hjollund *et al.*, 2000)	Occupational exposure to high temperatures	Insufficient database	Insufficient database
(Jockenhovel *et al.*, 1990)	Sauna sessions	Increased	Negative influence not sufficiently proven
(Watanabe, 1959)	Genital heat exposure in a water bath (>43 °C)	Increased	Impaired
(French *et al.*, 1973; Evenson *et al.*, 2000; Sergerie *et al.*, 2007)	Fever episodes (3 days, >39 °C)	Increased	Impaired
(Zorgniotti and Macleod, 1973; Jung *et al.*, 2002)	Varicocele	Increased	Conflicting results

2.2. Temperature and sperm chromatin integrity

Spermatogenesis is a complex and multi-step process which requires ~ 74 days in man for one cycle (Heller and Clermont, 1964). During this period DNA of germ cells is vulnerable to a range of insults and errors. DNA damage occurring in the male germ line is thought to contribute in birth and developmental defects and may have consequences for the later life of the offspring (Aitken and De Iuliis, 2007). The potential for insults to the integrity of sperm DNA acquired great concerns after the reports on transgenerational effects of radiation on murine spermatogenesis (Haines *et al.*, 2002). Although other factors such as chemotherapy, toxins or pathological conditions can affect sperm chromatin but higher testicular temperature is a factor which may contribute significantly to sperm DNA/chromatin alterations as demonstrated in several studies (Karabinus *et al.*, 1997; Sailer *et al.*, 1997; Ahmadi and Ng, 1999; Love and Kenney, 1999). In this section studies on scrotal or whole body hyperthermia and its consequences on sperm chromatin and male fertility are addressed.

2.2.1. Evidence from animal studies

2.2.1.1. Temperature and sperm chromatin/DNA abnormalities

Several animal studies have been reported on the effects of higher scrotal/whole body heating on sperm chromatin. For example, the scrotal regions of mice were exposed to different degrees of temperature i.e. 38, 40 and 42 °C for 60 minutes to investigate the effects of hyperthermia on sperm chromatin. Mice were sacrificed after the heat exposure and the changes recorded were found to be directly proportional to the degree of heat with maximum effects at 42 °C. A decrease in the testicular weight, testicular haploid cells and

an increase in the diploid cells was observed. Chromatin integrity assessed by SCSA was severely compromised at 42 °C and these changes were observed at all stages of spermatogenesis (Sailer *et al.*, 1997). However the temperature in this study was higher than the physiological temperature and the spermatozoa were obtained after killing the animals which could enhance the adverse effects. In experiments of Karabinus *et al.,* (1997) the scrotal insulation of Holstein bulls was obtained continuously for 48 hours (temperature achieved not reported), samples were collected after 3 days interval post scrotal insulation, freezed and thawed for sperm chromatin structure analysis by SCSA. The results showed decreased stability of bull sperm chromatin in the ejaculated spermatozoa which were in the testes at the time of heat induction. In another study mice were given a single heat shock by dipping in water bath after sedation at 43 °C for 20 minutes. Sperm DNA damage was analysed by TUNEL assay with a recorded damage as early as 8 h after heat treatment with expression of heat shock proteins Hsp 70-1 and Hsp 70-3 (Rockett *et al.*, 2001). Furthermore, a significant DNA damage of epididymal spermatozoa has been reported as early as 1 h post heat treatment (42 °C for 30 min) as assessed by COMET and sperm chromatin structure assay (SCSA) (Banks *et al.*, 2005). In a more recent study of Paul *et al.,* (2008b) male mice were subjected to a single heat stress at 38 °C, 40 °C and 42 °C for 30 minutes. Sperm chromatin structure assay performed on epididymal spermatozoa showed a temperature dependant increase in the number of sperm with impaired chromatin. The damage was slight at 38 °C while it was more profound at higher temperatures i.e. 40 °C and 42 °C. Moreover, percentage of TUNEL positive epididymal spermatozoa have been shown to be significantly higher in mice exposed to heat stress during 30 min at 42 °C compared with controls. Spermatozoa were recovered on 7, 14, 21, 28 or 60 days after heat stress. Maximum DNA damage was observed 14 days post treatment (Perez-Crespo *et al.*, 2008). These data reveal that alterations in sperm

parameters and DNA damage are proportionate with the degree of temperature. In conclusion the higher the degree of temperature the more damage on sperm chromatin.

With the back-ground of the data on heat stress and sperm chromatin alterations, the next question is whether the altered or fragmented sperm DNA has an impact on fertilization, embryo implantation or embryo development? In the next section these questions are addressed in the light of available evidences in literature.

2.2.1.2. Temperature, pregnancy rate and fate of embryo

The principal animal studies which have investigated the effects of heat on male fertility in terms of fertilization rates, pregnancy and embryo loss are given in Table 2 of this section. In view of the results of these studies it appears that increased testicular temperature (scrotal or ambient) results in the reduction or suppression of male fertility. For example several studies demonstrated a decrease in fertilization rates obtained with heated spermatozoa either by scrotal or whole body (ambient) heating (Dutt and Simpson, 1957; Fowler and Dun, 1966; Fowler, 1968; Braden and Mattner, 1970; Rathore, 1970; Wattemann *et al.,* 1979).

Other studies showed a decrease in the fertilization rates, retarded early embryo development or an increase in the rate of embryo loss in heated group (Rathore, 1968; Howarth, 1969; Burfening *et al.,* 1970; Wattemann *et al.,* 1976; Setchell *et al.,* 1988; Jannes et al., 1998). Similarly, a quite recent study in mice showed a reduction of 7% in pregnancy rates as well as retarded early embryo development and reduced litter size in females which were mated with males heated to 42 °C for 30 minutes (Paul *et al.*, 2008b)

However, there are studies which did not report a significant change in fertilization rates in heated group. For example Bellvé reported no change in fertilization rate but a retardation in embryo development (Bellve, 1972). Males heated to an ambient temperature of 34.5 °C

for 24 h were mated with normal females for six consecutive days (3 to 8 inclusive) after heat stress. A significant decrease was seen in eight-cell embryos at 54 h post fertilization (Bellve, 1972). Subsequently, in super ovulated females mated with heated males (ambient temperature of 34.5 °C for 24 h) an accumulation in morula stage embryo while a reduction in blastocyst was observed after 88 and 96 h of hCG injection (Bellve, 1973). Furthermore, in vitro culture of two-cell stage embryos sired by heated males showed significant blastocyst decrease after 120 h (Bellve, 1973). In later studies no change in fertilization rate was observed in heated group but higher embryo loss occurred compared to the control group (Mieusset *et al.,* 1992a). Semen samples from rams were collected, analyzed and frozen before heating and at days 4, 15 and 21 during heating. Ewes were inseminated with frozen semen and no differences in fertilization rates were recorded at any time in heated groups compared with controls, nor were there differences in the pregnancy rates (assessed at 17 days after insemination) at days 4 and 15 post heating (primarily epididymal spermatozoa). Nevertheless, as the pregnancy developed (between 17-65 days after insemination), abortion rates were higher at all times in heated groups than in controls (Mieusset *et al.*, 1992a).

Likewise, impaired *in vivo* embryo development was observed in mice when males were exposed to an ambient temperature of 36 °C for 24 h (Zhu and Setchell, 2004). Heat exposure at high degree (42 °C for 30 min) led to a marked decrease in the pregnancy rate and also change in the sex ratio of embryos fertilized with heat stressed epididymal spermatozoa (Perez-Crespo *et al.*, 2008). Moreover, Paul *at al.,* (2008b) found decreased pregnancy rate, placental weight and litter size in a group of normal female mice mated with heated males (scrotal heating, 42 °C for 30 min).

In conclusion heat stress adversely affects sperm quality and even if fertilization occurs the embryo development is impaired which highlights the importance of the quality of sperm chromatin to achieve a successful pregnancy.

Table 2.1. Principal animal studies on heat stress and male fertility

Authors	**Species**	**Heating duration**	**Heating method**	**Fertilization Rate**	**1st Pregnancy Diagnosis**	**2nd Pregnancy Diagnosis**	**Early embryo development/loss**
Dutt and Simpson, 1957	Ram (South down)	112d	T°1=26.1°-31.7° (ambient) T°2=7.2°-8.9° (air conditioned)	C=13% H=50%	3 days post coitus	/	T°1=69% T°2=41%
Howarth *et al.*, 1965	Rabbit (New Zealand)	6-8h	C=21° H=32° (rabbit uteri)	C=80% H=83%	30 h post coitum	12 d post coitum	Survival pre-implantation C=94% H=72%
Fowler and Dun, 1966	Ram (PL/PP)	21d 3d	H=26.7°-36.1° (ambient) T°=14.5-17.2°	T°=43% H=18% T°=50% H=21%	Slaughtered 30 d post artificial insemination	/	/
Rathore, 1968	Ram (PL/PP)	8h/d for 2d 8h/d for 4d	H=40.5° (ambient) C= room °T	C=70% H=45% H=15%	12 days post coitus	40 d post heat	H1=80% H2=100% C=10%

C = control, H = heat, °T = temperature, d= day, h =hour

Table 2.2. Principal animal studies on heat stress and male fertility

Authors	**Species**	**Heating duration**	**Heating method**	**Fertilization Rate**	**1st Pregnancy Diagnosis**	**2nd Pregnancy Diagnosis**	**Early embryo development/loss**
Fowler, 1968	Ram (PL/PP)	2h	Local scrotal H=39.5°-42° C= room °T	C=69% H=42%	Slaughtered 3rd week post artificial insemination	/	/
Burfening and Ulberg, 1968	Rabbit (New Zealand)	3h	C=21° H1=38° H2=40° (Spem incubation)	C=98% H1=98% H2=96%	30h post coitum	9d post coitum	Pre-implantation survival C=62.5 H1=64.3 H2=42.2
Howarth, 1969	Ram (?)	4d	H=32° (ambient) C= room °T	**1st week** C=88%, H=62% **2nd week** C=100%, H=0 **3rd week** C=100%, H=0	30-40h post heat	34d	**1st week** C=13% H=50% **2nd week** C=0% H=67% **3rd week** C=40% H=100%

C = control, H = heat, °T = temperature, d= day, h =hour

Table 2.3. Principal animal studies on heat stress and male fertility

Authors	**Species**	**Heating duration**	**Heating method**	**Fertilization Rate**	**1st Pregnancy Diagnosis**	**2nd Pregnancy Diagnosis**	**Early embryo development/loss**
Rathore, 1970	Ram (PL/PP)	1d 2d 3d 4d	H=40.5° (ambient) C= room °T	C=93% 1d=66% 2d=42% 3d=23% 4d=7%	Slaughtered 60-70h post copulation	/	/
Burfening *et al.*, 1970	Mice (ICR)	24h	H=32° (ambient) C=21° (ambient)	**1-5d post heat** C=87% H=78% **6-10d post heat** C=83% H=62% **11-15d post heat** C=77% H=42% **16-20d post heat** C=92% H=11% **21-25d post heat** C=85% H=52% **26-30d post heat** C=89% H=76%	48h after vaginal plug	10d post plug	**1-5d** C=34% H=53% **6-10d** C=31% H=70% **11-15d** C=36% H=79% **16-20d** C=28% H=88% **21-25d** C=33% H=64% **26-30d** C=20% H=47%

C = control, H = heat, °T = temperature, d= day, h =hour

Table 2.4. Principal animal studies on heat stress and male fertility

Authors	**Species**	**Heating duration**	**Heating method**	**Fertilization Rate**	**1st Pregnancy Diagnosis**	**2nd Pregnancy Diagnosis**	**Early embryo development/loss**
Bellve, 1972	Mice (ICR)	24h	34.5°	NS	54h after vaginal plug	10 days of gestation	Reduced implantation sites and viable foetus
Wettemann *et al.*, 1979	Boar (Yorkshire)	8h (daily for 90d) 16h (daily for 90d)	C=23° 8h=34.5° 16h=31°	C=82% 8h+16h=59%	Slaughtered 30d post insemination	/	C=18% 8h+16h=22%
Setchell *et al.*, 1988	Rat (Porton-wistar)	30min	H=43° local C=?	C=91% H=54%	Next day post- mortem after vaginal plug		Embryo degenerated C=0% H=53%
Mieusset *et al.*, 1992a	Rams (Merino Australian)	16h daily for 21d	Scrotal heating H=1.2°-2.2° (above testes °T) C= room °T	NS	17 days post insemination	65 days post insemination	Higher embryo loss in heated group

C = control, H = heat, °T = temperature, d= day, h =hour

Table 2.5. Principal animal studies on heat stress and male fertility

Authors	**Species**	**Heating duration**	**Heating method**	**Fertilization Rate**	**1st Pregnancy Diagnosis**	**2nd Pregnancy Diagnosis**	**Early embryo development/loss**
Jannes *et al.*, 1998	Mice (C57BL)	20 min	Local testes heating H=40° C=33°	C=65.1% H=44.9%	/		Retarded early embryo development and reduced embryo weight in heated group
Setchell *et al.*, 1998	Mice (5WARI) (Balb-C) (F1=CBA male×C57 female)	H1=20min H2=20min H3=30min	H= 42° scrotal heating C=33° scrotal heating	/	/		Reduced embryo size 4-35 days post heat at 10.5 day of gestation in H1+H2+H3
Zhu and Setchell, 2004	Mice (B6CBF1)	24h	H=36° (ambient) C=room °T	/	Embryos were collected 14-16h, 34-39h or 61-65h after mating or from the uterus at 85-90h after mating		Reduced embryo development compared to control

Table 2.6. Principal animal studies on heat stress and male fertility

Authors	Species	Heating duration	Heating method	Fertilization Rate	1st Pregnancy Diagnosis	2nd Pregnancy Diagnosis	Early embryo development/loss
Paule *et al.*, 2008b	Mice C57BL/6	30 min	H1=38°, H2=40° H3=42° C= room °T	H3=7% reduction H1+H2=NS	killed at 14.5d and at 3.5d		Retarded early embryo development and reduced litter size
Pérez-Crespo *et al.*, 2008	Mice (CD1)	30 min	H=42° (ambient) C=33°C (ambient)	?	Killed at 14d of pregnancy Number of live foetuses and resorption sites noted		Heat stress led to foetal sex ratio distortion

C = control, H = heat, °T = temperature, d= day, h =hour (Updated from PhD thesis "effets de la témperature sur les fonctions testiculaires et epididymaires" by Dr Roger Mieusset, 1992).

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2.2.2. Human studies

2.2.2.1. Temperature and sperm DNA/chromatin alterations

In men, to date, only case reports have been documented on higher testicular temperature and its consequences on sperm chromatin integrity. The first case report was of a fertile patient presenting with an episode of influenza and high fever (39-40 °C) about a decade ago (Evenson *et al.*, 2000). Semen samples were obtained and analyzed at 18-66 days post fever to evaluate sperm chromatin integrity. The results of SCSA at 18 day post fever revealed that 36% of sperm demonstrated denatured DNA which decreased to 23% by 39 day post fever. In the second case report semen samples were collected from a fertile donor before the onset of an episode of fever (39-40 °C) and at days 15, 37, 58, 79 and >180 post fever. The SCSA results showed 24% and 36% sperm DNA fragmentation at 15 and 37 days post fever respectively which then decreased to 15% and 8% at days 58 and 79 post fever respectively (Sergerie *et al.*, 2007).

2.2.2.2. Temperature, pregnancy rate and fate of embryo

During our literature search we could not find a single human study on the effect of high testicular temperature and pregnancy outcome.

2.2.2.3. Male mediated heat stress

On the other hand there is a concern that parental life style like occupation, smoking and late desire of parenthood also cause an alteration in the gamete genomic integrity which may have bad consequences on the offspring (Crow, 1995, 1997; Hemminki and Kyyronen, 1999; Hemminki *et al.*, 1999; Zenzes *et*

al., 1999; Zenzes, 2000). The effects of increasing paternal age on the child health have also been documented. A variety of genetic diseases has been attributed with increased paternal age (Crow, 1995, 1997). De La Rochebrochard and Thonneau reported that with increasing paternal age (40 years), the rate of fertilization decreases while frequency of miscarriages increases (De La Rochebrochard and Thonneau, 2005). In a more recent study a significant decrease in the blastocyst formation and live birth rates have been shown where males were more than 50 years old. However, the implantation and pregnancy rates were not affected (Frattarelli *et al.*, 2008).

In view of the data from animal models presented in the literature review section the fact is established that temperature increase causes sperm chromatin alterations, impairs embryo development as well as results in embryo loss. Due to the lacking evidence in men, the question of similar effects on human sperm chromatin integrity needs to be addressed because testicular hyperthermia has been proposed as a contraception method in men. Therefore, investigating the consequences of hyperthermia on sperm chromatin specifically during inhibition or recovery phases of spermatogenesis under such method was of high interest. In this context, to answer the question, if mild increase in testicular temperature alters sperm chromatin integrity, we worked on human model of heat associated contraception introduced by our team (Mieusset *at al.,* 1985).

STUDY

1. STUDY OBJECTIVES

1.1. Principal objective

To investigate the effects and dynamics of a mild induced testicular and epididymal hyperthermia on sperm DNA integrity in men by sperm chromatin structure assay (SCSA).

1.2. Secondary objectives

To analyse the effects of hyperthermia on sperm chromatin integrity by acid aniline blue staining and on the dynamics of alterations and recovery of sperm parameters essential for normal fertilization such as: sperm motility, vitality, morphology and concentration.

2. MATERIALS AND METHODS

2.1. Study design

This is a prospective and longitudinal experimental study performed on health and fertile volunteers. The analysis were carried out on semen; before, during and after diurnal artificial cryptorchidism (DAC) i.e. induced testicular and epididymal hyperthermia (Mieusset *et al.*, 1987a; Mieusset and Bujan, 1994). The study protocol was approved by the Toulouse Ethics Committee (Comité Protection des Personnes Sud-Ouest et Outre Mer I), France and was supported by AOL (09 16 102) CHU Toulouse, France.

2.2. Recruitment of volunteers

An advertisement was given in the press and University Hospital Paule de Viguier, Toulouse, France, to attract the volunteers. Following the announcement we made a list of all the volunteers who contacted us. Among the registered volunteers five men (age 25-35 years) were selected on the basis of following criteria. All participants gave written signed consent before the start of experimentations.

Inclusion criteria:

- Adult men in good health
- Have fathered at least one child
- Without any adrological or inguinal history
- Normal clinical examination
- Testicles could be maintained at inguinal position
- Agreed to adopt contraception during 12 months of study period (male preservatives or female contraception)
- Posses social security

Exclusion criteria

- Men with any andrological history
- Presenting with a disease
- Presenting or with history of inguinal hernia
- Anomalies of andrological examination
- Presence of hyperthermia during last 3 months (before inclusion in the study)
- Had consulted for infertility
- Refused to adopt contraception during the study period
- Persons without legal autonomy
- Participating in an other research during the inclusion period

2.3. Method of testicular and epididymal hyperthermia

We used a diurnal artificial cryptorchidism method to induce testicular and epididymal hyperthermia. The method was developed by our group (Mieusset *et al.*, 1985) around 30 yeas ago, is well tolerated and reversible. Each volunteer was provided specific underwear. A comprehensive demonstration was given to the volunteers explaining how to push up and maintain the testicles at the upper part of the root of the penis (Fig 11). All volunteers were able to push and maintain the testicles easily in this position. The testicles were pushed up into the inguinal canals and maintained there daily for waking hours (15h±1) for 120 consecutive days. The support to the testes was ensured by the briefs (underwear) provided with an orifice allowing the penis and scrotum to be exteriorized and specifically made for each volunteers (six/volunteers).

2.3.1. Justification of the model

The model used has been well described in men which is, non hormonal, efficient enough to induce reversible alterations in spermatogenesis and has been used as male contraception. In healthy men this protocol has been already used for longer periods (>12 months) (Mieusset *et al.*, 1985; Mieusset *et al.*, 1987a; Shafik, 1991a, 1992; Mieusset and Bujan, 1994).

Key points of the model

Our model has two important features:

a) Achieved testicular and epididymal temperature was less than core body temperature (~ 36-36.5 °C)

b) The heat source was human body itself: (1) scrotum was excluded from the physiological thermoregulation process and, (2) testes were lifted up in a non-physiological body location where environmental temperature was higher than physiological temperature in scrotum but lesser than the whole body temperature.

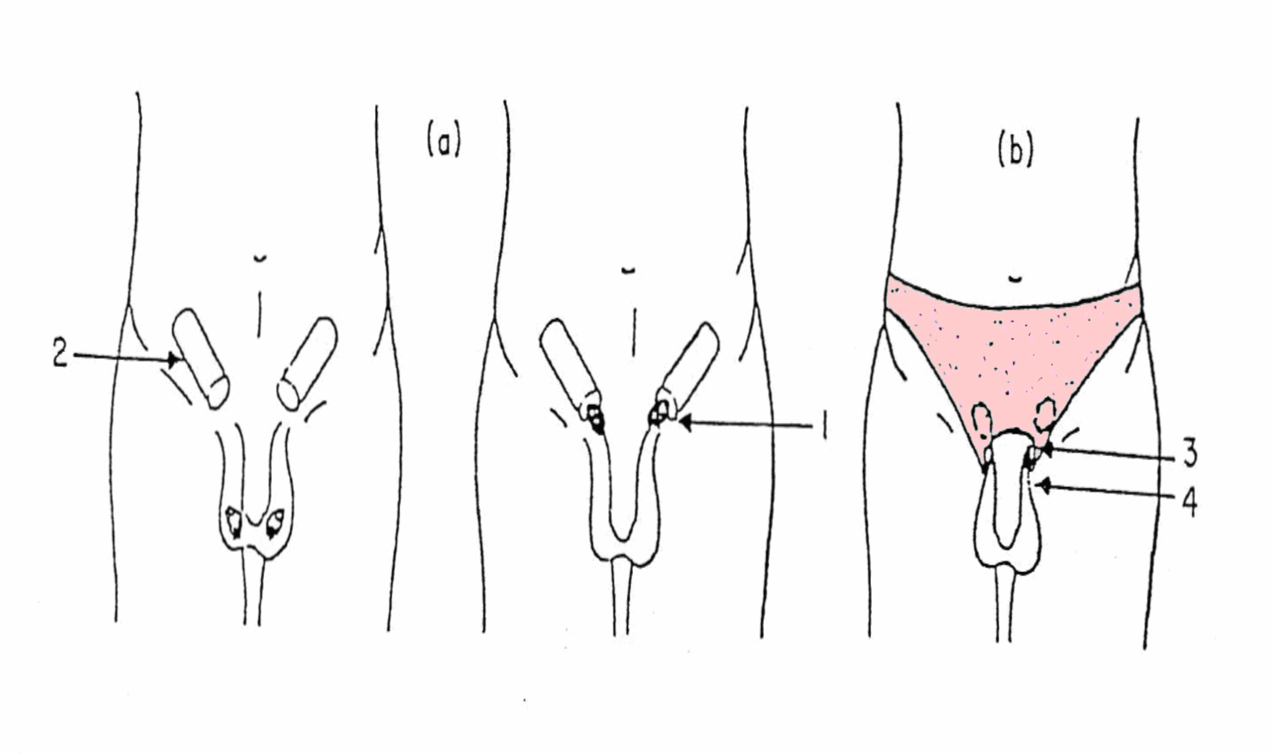

Figure 11. Schematic representation of testicular and epididymal hyperthermia induction (a) testes are lifted up (1) close to the inguinal canal (2). (b) Testes are maintained in the previous location by means of underwear in which a hole (3) was made at the level of the root of the penis. The penis and the scrotal skin are passed through this hole (4) (Mieusset *et al.,* 1994).

2.4. Chronology of explorations

We followed a pattern of semen collections and clinical explorations according to the chronology of spermatogenesis as described by Heller and Clermont (1964) and epididyaml transit timing reported by Rowley *et al.,* (1970), lately used by Da Cunha *et al.,* (1982) and May *et al.,* (2000) (Fig 12).

Chronology of semen sampling

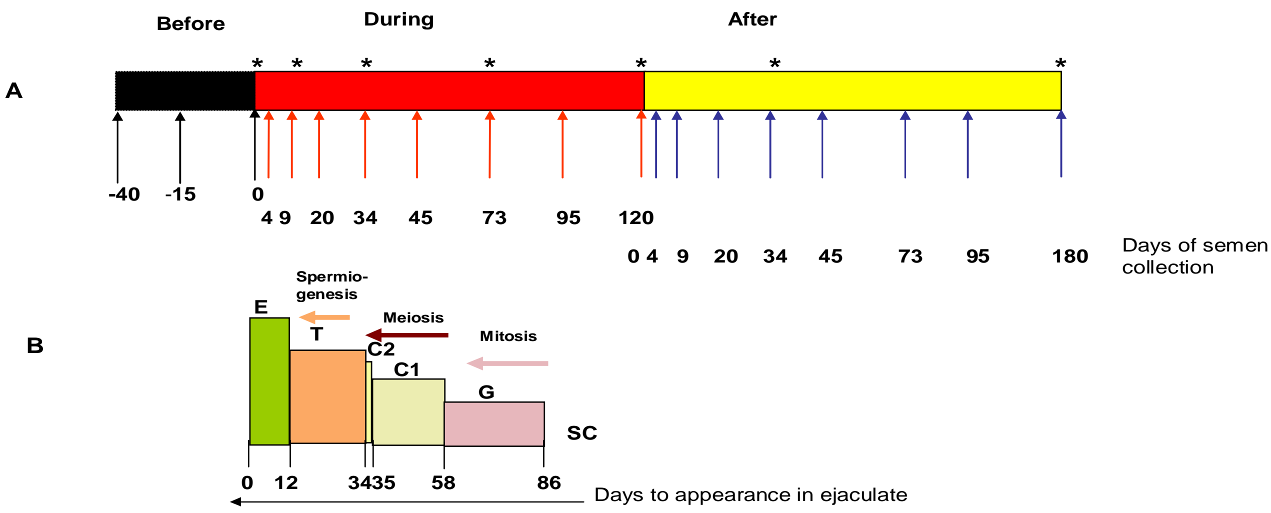

Figure 12. Chronology of semen sampling: **(A)** Schematic representation of semen sampling timing during the three study periods, before, during and after mild induced testicular and epididymal hyperthermia in men. * Represents the days when volunteers underwent complete clinical evaluations during the three study periods. **(B)** Represents the location and evolutionary stages of sperm during the spermatogenic process at induction of hyperthermia (D0) and their expected appearance in ejaculates. The bars represent E epididymal sperm, T spermatids, C2 spermatocytes II, C1 spermatocytes I, G spermatogonia, SC stem cells (modified from May *et al., 2000*).

2.4.1. Clinical examinations/consultations

Before hyperthermia

Two consultations were performed before starting hyperthermia in which complete clinical examination and semen analysis were done. The first, 40 days before beginning of hyperthermia and the second on the day of hyperthermia induction (Table 3).

During hyperthermia

In order to ensure the efficacy and comfort of the technique complete clinical examination of volunteers was performed at: D9, D34, D73 and D120 during hyperthermia (Table 3).

After hyperthermia

Complete clinical examination was performed at D34 and D180 to ensure the efficacy and reversibility of the technique (Table 3).

2.4.2. Semen collection

Before hyperthermia

Two semen samples were collected from all participants: first at 40 days (D) and second at 15 days before induction of hyperthermia (Fig 11).

Day of induction of hyperthermia

Third semen sample was collected at day 0 (D0) before wearing the underwear. This semen sample also corresponds to the before hyperthermia period. After semen collection the

testicles were pushed and maintained at inguinal positions for 15 h ±1 for 120 consecutive days (Fig 11).

At D0 blood samples were also taken for serology and hormonology analysis.

During hyperthermia

According to the spermatogenesis and epididymal transit timings in human (Heller and Clermont, 1964; Rowley *et al.,* 1970; May *et al.*, 2000), a sampling chronology was determined and eight semen samples, at D4, D9, D20, D34, D45, D73, D95 and D120, were collected during hyperthermia (Fig 11).

After hyperthermia

Eight semen samples were collected after the cessation of hyperthermia at D4, D9, D20, D34, D45, D73, D95 and D180.

Table 3. Chronology of consultations, semen samplings and principal tests applied.

	Before			**During hyperthermia**							
Semen collection days	**<-10-40**	**-15**	**0**	**4**	**9**	**20**	**34**	**45**	**73**	**95**	**120**
Semen sampling	*	*	*	*	*	*	*	*	*	*	*
Consultations	*	*	*		*		*		*		*
Volunteer's consentement		*									
Sperm DNA Fragmentation	*	*	*	*	*	*	*	*	*	*	*

	After hyperthermia							
Semen collection days	**4**	**9**	**20**	**34**	**45**	**73**	**95**	**180**
Semen sampling	*	*	*	*	*	*	*	*
Consultations				*				*
Sperm DNA Fragmentation	*	*	*	*	*	*	*	*

2.5. Sample collection and processing

Three semen samples were collected before, 8 during and 8 after hyperthermia by masturbation in our laboratory after a mean sexual abstinence duration of 4.1 (± 0.1) days. At all data points samples from 5 volunteers were collected, except at D95 during hyperthermia when one volunteer did not attend for family reasons. After liquefaction (30 minutes at 37 °C), semen analysis was performed according to WHO laboratory manual guidelines (WHO, 1999). All readings were taken within one hour of sample collection.

2.5.1. Semen volume

Semen volume was measured by a graduated pipette and was noted in millilitres. The pH was measured with reaction paper.

2.5.2. Sperm motility

Motility was observed under an optical microscope. A drop of semen (10 µl) was put on the glass slide with the help of a measuring pipette. A cover slide (20×22 mm) was placed on the drop and was allowed to settle down. The sperm motility was assessed as follows:

1) rapid progressive spermatozoa as: a
2) progressive spermatozoa as: b
3) motile but non progressive as: c
4) immotile spermatozoa as: d

2.5.3. Sperm and round cell counts

Sperm and round cell counts ($\times 10^6$/ml) were performed on Malassez cells (Rogo Sanlab Arcueil, France). Depending upon the concentration of spermatozoa, according to days of

study periods, dilutions of 1/10, 1/20 or 1/50 were used, already prepared and routinely used at CECOS Midi-Pyrenees.

In majority a 1/20 dilution was used i.e. a semen volume of 50 µl was taken into a small glass tubes. In the tube 950 µl of dilution solution was added and was mixed with the help of a vortex. The procedure was performed in duplicate. A Pasteur pipette was added in the tube so that the solution rises by capillary action. With the help of a Pasteur pipette solution was added on both sides of the counting chamber (Malassez cells, Rogo Sanlab Arcueil, France).

Sperm count calculations were expressed in millions/ml

In case of 1/20 dilution

$(X1 + X2) \times 20 \times 50 \times 1000 = (X1 + X2) \times 1,000,000$

As X1 = square 1

X2 = square 2

20 = dilution factor

50 = to convert the volume to mm^3

1000 = to convert volume from ml to cm^3 as 1 square = $1/100^{th}$ of whole cell

When instead of two squares (X1 + X2) one band was counted the calculation was made as follows:

$X \times 20 \times 10 \times 1000 = X \times 200,000$ (as 1 band = $1/10^{th}$ of whole cell).

2.5.4. Sperm viability

Sperm viability was assessed by staining the spermatozoa with Eosine (Sigma E 6003) and Nigrosine (Sigma 4754) as routinely used at CECOS Midi-Pyrenees. A semen volume of 10 µl was transferred in an eppendorf. In the same eppendorf 20 µl of stain Eosine was added, gently mixed, and after 30 seconds 30 µl of Nigrosine was added. After mixing a drop of 10

µl was placed on a glass slide and observed under oil immersion objective (× 100) of phase contrast microscope.

Live cells appeared white (unstained) while dead cells appeared red in the head regions. A minimum of 100 spermatozoa per slide were calculated except the days when sperm count was very low > 50 sperm cells were counted.

2.5.5. Sperm chromatin structure assay (SCSA)

Sperm DNA fragmentation index (DFI) and high DNA stainability (HDS) were measured by conventional SCSA techniques (Evenson *et al.*, 2000; Evenson *et al.*, 2002) routinely used in our laboratory (Sergerie *et al.*, 2007; Pecou *et al.*, 2009). A fraction of semen sample containing a minimum of 4×10^6 spermatozoa was separated from the original sample. For sperm fixation, 5 ml of Dulbecco's phosphate buffer saline (DPBS pH 7.4, 1X, GIBCO, Auckland, New Zealand) was added drop by drop to 1 ml, the remaining 4 ml were then added more rapidly and centrifugation was performed at 630*g* for 10 minutes. Supernatant was removed and the precipitate was mixed in 1 ml of DPBS. Drop by drop, 4 ml of paraformaldehyde (PFA 1%, Merck >95%, NaOH, PBS, pH 7.4) was added in the sample tube to a final volume of 5 ml and was incubated at room temperature for 30 minutes. A second centrifugation was carried out at 1500*g* for 10 minutes, the supernatant was discarded and the precipitate was resuspended in the required volume of PBS according to sperm count. A volume containing a minimum of 2×10^6 spermatozoa was drawn from the sample already fixed in PFA and centrifuged at 1500*g* for 10 minutes. The precipitate was resuspended in 200 µl of DPBS and transferred into a cytometric tube with identity of the sample. Samples were run in duplicates at all data points except when sperm count dropped lower than the required sperm number for SCSA analysis. The flow cytometer was pre-equilibrated by passing a tube containing 1.2 ml of acridine orange and 0.4 ml of acid detergent (pH 1.2) for 5 minutes.

After vortexing, 0.4 ml of acid detergent was added to the sample and mixing was continued by gently shaking the tube for 30 seconds. Then 1.2 ml of acridine orange was added and incubated on ice for 3 minutes. After incubation the sample was passed through the cytometer and at least 5000 sperm cells were counted.

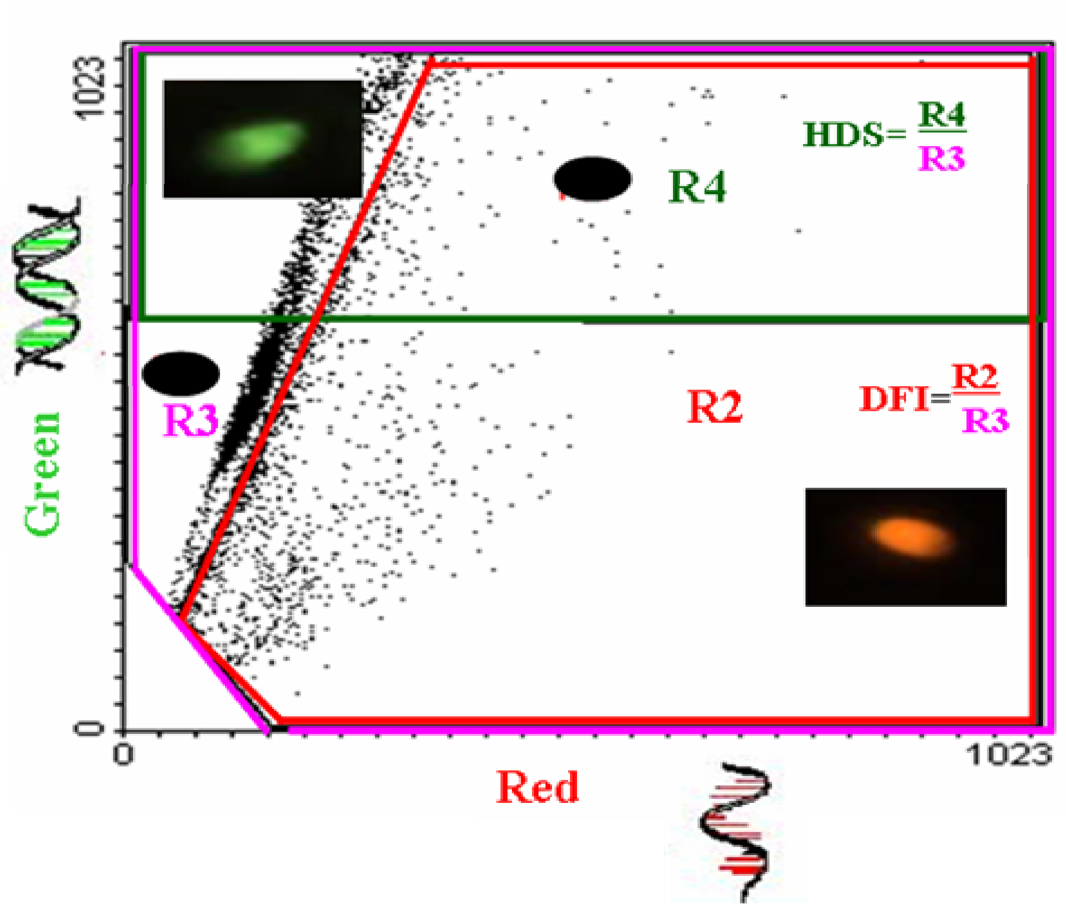

Figure 13. Representation of the SCSA analysis: Y-axis shows double stranded DNA giving green flourescence; X-axis shows single stranded DNA giving red fluorescence. The zone R3 is the total sperm population, R2 is the sperm population with fragmentated DNA (DFI) and R4 is the population with high DNA stainability giving strong green flourescence (HDS).

2.6. Statistics

Means (± SEM) of the three means (± SEM) of the three data points (each data point $n = 5$) before hyperthermia were compared with the means of each data point ($n = 5$) during and after hyperthermia for all semen parameters (significance $p<0.05$). Mean values of sperm viability, motility, total sperm and round cell count, sperm DFI and HDS were compared by applying non-parametric Wilcoxon's test using STATA software version 8.

3. Results

The results of the study are presented in three segements:

1. Sperm chromatin structure assay (SCSA)

2. Sperm morphology

3. Acid aniline blue test

3.1. Sperm Chromatin

3.1.1. Article: **Mild induced testicular and epididymal hyperthermia alters sperm chromatin integrity in men**

3.1.2. Summary

In this part, results of experimental protocol on sperm chromatin and sperm parameters are presented. The results obtained were divided into two segments for easy interpretation of the data:

a) Testicular and epididymal hyperthermia and sperm chromatin integrity
b) Testicular and epididymal hyperthermia and sperm characteristics (count, motility, viability) including round cells.

Sperm chromatin

Sperm DFI was significantly increased ($p<0.05$) at day 20 (D20) (16.7±3.9), D34 (23.8±2.9) and D45 (31.3±5.4) during hyperthermia compared with control (before hyperthermia) (11.9±1.5). Sperm HDS started to increase as early as D20 (7.4±1.5) during hyperthermia and was significantly higher compared to control (5.9±0.3), at D34 (10.9±1.0) and D45 (13.0±1.1) respectively. Both sperm DFI and HDS remained higher compared with respective controls throughout the entire period of hyperthermia. At D73 after cessation of hyperthermia, sperm DFI/HDS returned to control values.

Sperm parameters

Our results demonstrated a significant decrease in sperm count and total count as early as D34 during hyperthermia which remained persistently low compared with control (before hyperthermia) throughout the entire period of hyperthermia. Sperm count per millilitres before hyperthermia was $77.7 \pm 8.1 \times10^6$ which reduced to $15\pm3.1 \times10^6$ at D34, $2.8\pm1.0 \times10^6$ at D45 and remained less then one million ($<1\times10^6$) till the end of hyperthermia period. Total sperm count before hyperthermia was 315.2 (±19.4) $\times10^6$ which significantly decreased to 56.0 (±13) $\times 10^6$ at D34 during hyperthermia and further dropped to 16.0 (±8) $\times 10^6$ (~95%

decrease compared with control) at D45 and was $\leq 2 \times 10^6$ till the end of hyperthermia period. One man presented azoospermia at D95, another at D120 during hyperthermia and the remaining had rare spermatozoa in the ejaculates.

Percentage of motile spermatozoa before hyperthermia was 47 ± 1.0 which significantly decreased to 31± 2.9 at D20 and remained low compared with control, till the end of hyperthermia. Percentage of viable spermatozoa before hyperthermia was 73.2 ± 1.7 which decreased to 49±9.2 at D34 and remained lower till the end of the hyperthermia period.

Total round cell count before hyperthermia was $1.43 \pm 0.26 \times 10^6$ which started to increase at D20 ($6.3 \pm 1.7 \times 10^6$) and was significantly higher at D34 ($6.0 \pm 1.9 \times 10^6$) then returned to control values during whole hyperthermia period with exception of lower significant value at D73.

At D73 after cessation of hyperthermia, all sperm parameters returned to respective control values.

Running title: **Testes hyperthermia and sperm chromatin**

Title: **Mild induced testicular and epididymal hyperthermia alters sperm chromatin integrity in men**

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Under review: Fertility and Sterility

Capsule: Mild induced testicular and epididymal hyperthermia in men caused drastic and reversible effects on sperm chromatin integrity which appeared before any changes in sperm output.

Abstract

Objective: To investigate the effects of a mild induced testicular and epididymal hyperthermia (+2°C) on sperm chromatin integrity in men.

Design: Experimental prospective study.

Settings: University Hospital.

Patient(s): Five healthy fertile volunteers.

Intervention(s): Testicular and epididymal hyperthermia was induced by maintaining the testes at inguinal position with the support of specially designed underwear 15±1h daily for 120 consecutive days.

Main Outcome Measure(s): Classical semen characteristics. Sperm DNA fragmentation index (DFI) and high DNA stainability (HDS) were analysed by sperm chromatin structure assay (SCSA).

Result(s): Compared with baseline values, sperm DFI and HDS were significantly increased as early as day (D) 20 and D34 respectively and remained elevated during the entire period of hyperthermia. Percentages of motile and viable spermatozoa decreased as early as D20 and D34 respectively while total sperm count decreased at D34 during hyperthermia and remained low during the entire hyperthermia period. All studied parameters returned to respective baseline values at D73 after cessation of hyperthermia.

Conclusion(s): Mild induced testicular and epididymal hyperthermia largely impaired sperm chromatin integrity which appeared before any changes in sperm output. These findings may have clinical implications in male contraception, infertility and assisted reproductive technology.

Key Words: Testis, sperm chromatin, infertility, temperature, humans

INTRODUCTION

Heat stress has deleterious effects on testicular functioning and is a well-known cause of impaired spermatogenesis (1). Several studies in different animal species, for example in mice (2, 3), rats (4, 5) and rams (6), have shown harmful effects of induced testicular hyperthermia on sperm characteristics. Testicular and epididymal hyperthermia also has undesirable effects on the epididymis functions causing rapid sperm epididymis transit resulting in a decreased number of motile and mature ejaculated spermatozoa (7, 8).

Several endogenous factors can modify human testicular temperature. For example, cryptorchidism in children (9), and in infertile men with a history of cryptorchidism (10, 11). Varicocele can also disturb testicular thermoregulation causing scrotal hyperthermia and alteration in sperm parameters (1, 12, 13). Further, an episode of fever in a man with body temperature 39-40 C° for 2 days caused reduced sperm output, motility and viability (14). Besides, exogenous factors have also been reported to increase scrotal temperature, such as sitting or sleeping postures (15-19), clothing (20), sauna baths (21), driving for long periods (22, 23) and occupational exposure to high environmental temperatures (24).

Knowing the potential effects of heat stress on spermatogenesis, some studies have been conducted on male contraceptive methods using different approaches to increase testicular temperature. Previously, we have created a diurnal artificial cryptorchidism (DAC) method to induce mild testicular and epididymal hyperthermia (36.5 C°) inferior to body temperature, which has shown a decrease in total sperm output (25) and sperm motility, and an increase in morphologically abnormal spermatozoa (26). This type of method was later used as a contraceptive method in men (27-30).

Moreover, testicular heat stress showed damaging effects on sperm DNA quality, causing arrest in early embryo development and high miscarriage rates as reported in animal studies. Mieusset et al. (31) observed no changes in sperm viability or motility in rams after 4

and 15 days of diurnal scrotal insulation, but there was an increase in embryo loss that may suggest possible harmful effects of heat stress on sperm genetic and/or epigenetic information. In mice, a paternal effect on embryo development has been demonstrated after a 24h exposure to an ambient temperature of 36 C° resulting in impaired embryo development (32). Besides, scrotal local heating of male mice to a high temperature (40-42C° for 30 minutes) caused sperm DNA breakdown, reduced pregnancy rates, low placental weight and litter size as well as arrested embryo development at blastocyst stage (33, 34).

In men, rare studies have examined the effect of testicular heat stress on sperm DNA quality. Evenson et al. (35) described the case of a patient whose sperm showed a high DNA fragmentation index (DFI) and altered composition of nuclear proteins after an episode of influenza and high fever (39.9 C°). Likewise, our group reported another case of high fever (39-40 C°) in a man resulting in a post-fever increase in DNA fragmentation index which returned to baseline values 58 days after fever (14).

Although studies on animal models have provided solid evidence on sperm DNA damage after heat exposure, no study in men has yet documented such damage during mild induced testicular and epididymal hyperthermia. For the first time in men, we report in the present study the deleterious effects of diurnal mild testicular and epididymal hyperthermia (at a temperature inferior to core body temperature) on sperm DNA quality.

MATERIALS AND METHODS

Study Population

The study was approved by the Toulouse Ethics Committee (Comité de Protection des Personnes Sud-Ouest et Outre Mer I), France. Five healthy fertile volunteers (age 25-35 years, having fathered at least one child) with no andrological, medical or surgical history were recruited after their written informed consent.

Study Design

The study was divided into three periods: before (baseline/control), during and after hyperthermia. The days of semen collection were established in accordance with the physiological chronology of spermatogenesis and epididymal transit (36-38) (Fig. 1).

Method of Testicular and Epididymal Hyperthermia Induction

We used the diurnal artificial cryptorchidism (DAC) method developed by our group (25), which is well tolerated and reversible. Each participant was provided with specially designed underwear with an orifice allowing the penis and the scrotum to be exteriorized. A comprehensive demonstration was given to the volunteers explaining how to push up and maintain the testicles at the upper part of the root of the penis. All volunteers were able to push and maintain the testicles in this position during waking hours (15 ± 1 h daily) for 120 consecutive days during hyperthermia. Regular clinical examinations were performed on different days (D) during and after hyperthermia (Fig. 1) by two andrologists (RM and LB).

Semen Sampling

Three semen samples were collected before, 8 during and 8 after hyperthermia. At all data points samples from 5 volunteers were collected, except at D95 during hyperthermia when

one volunteer did not attend for family reasons. Samples were collected by masturbation in our laboratory after a mean sexual abstinence duration of 4.1 (± 0.1) days. After liquefaction (37 °C for 30 minutes), semen analysis was performed as previously published (14, 39). Briefly, semen volume was measured by a graduated pipette and the pH was measured with reaction paper. Sperm motility was observed under optical microscope and expressed as percentage progressive motility (sperm crossing the microscopic field). Sperm viability was assessed by nigrosine and eosine staining (Sigma Aldrich France) and expressed as percentage viability. Sperm and round cells counts ($\times 10^6$) were performed on Malassez cell (Rogo Sanlab Arcueil, France). Total sperm and round cells counts were calculated per ejaculate (i.e. sperm/round cells count × semen volume). All readings were taken in duplicate within one hour of sample collection.

Sperm Chromatin Structure Assay (SCSA)

Sperm DNA fragmentation index (DFI) and high DNA stainability (HDS) were measured by conventional SCSA techniques (35, 40) routinely used in our laboratory (14, 39). Briefly, a fraction of semen sample containing a minimum of 4 × 106 spermatozoa was separated from the original sample. For sperm fixation, 5 ml of Dulbecco's phosphate buffer saline (DPBS pH 7.4, 1X, GIBCO, Auckland, New Zealand) was added drop by drop to 1 ml, the remaining 4 ml were then added more rapidly and centrifugation was performed at 630 g for 10 minutes. The supernatant was removed and the precipitate was mixed in 1 ml of DPBS. Drop by drop, 4 ml of paraformaldehyde (PFA 1%, Merck >95%, NaOH, PBS, pH 7.4) was added in the sample tube to a final volume of 5 ml and was incubated at room temperature for 30 minutes. A second centrifugation was carried out at 1500 g for 10 minutes, the supernatant was discarded and the precipitate was resuspended in the required volume of DPBS according to sperm count. A volume containing a minimum of 2 ×106 spermatozoa was drawn from the

sample already fixed in PFA and centrifuged at 1500 g for 10 minutes. The precipitate was resuspended in 200 µl of DPBS and transferred into a cytometric tube with identity of the sample. Samples were run in duplicates at all data points except where sperm count dropped lower than the required sperm number for SCSA analysis. The flow cytometer was pre-equilibrated by passing a tube containing 1.2 ml of acridine orange and 0.4 ml of acid detergent (pH 1.2) for 5 minutes. After vortexing, 0.4 ml of acid detergent was added to the sample and mixing was continued by gently shaking the tube for 30 seconds. Then 1.2 ml of acridine orange was added and incubated in ice for 3 minutes. After incubation the sample was passed through the cytometer and at least 5000 sperm cells were counted.

Statistical Analysis

Means (± SEM) of the three means (± SEM) of the three data points (each data point $n = 5$) before hyperthermia were compared with the means of each data point ($n = 5$) during and after hyperthermia for all semen parameters (significance $p<0.05$). Mean values of sperm viability, motility, total sperm and round cells counts, sperm DFI and HDS were compared by applying Wilcoxon's test using STATA software version 8.

RESULTS

No volunteer withdrew from the study due to discomfort, pain or any other medical reasons. No change was observed in mean (± SEM) semen pH (data not shown), semen volume, or duration of abstinence (Table 1) during the three periods of the study.

During Hyperthermia

Sperm motility, viability and count

Percentages of motile and viable sperm decreased significantly ($p<0.05$) compared with baseline values as early as D20 and D34 respectively and the difference remained significant until D120 (Table 1). Sperm concentration/ml decreased significantly as early as D34 and remained low during the entire period of hyperthermia (Table 1). Baseline total sperm count was 315.2 (± 19.4) $\times 10^6$, which significantly decreased to 56.0 (± 13) $\times 10^6$ at D34 and to 16.0 (± 8) $\times 10^6$ at D45 (95% decrease in sperm output). One of the five volunteers presented with azoospermia at D95, a second man at D120 and the remaining men had rare spermatozoa in their ejaculates at D120 (Fig. 2B).

Round cells

Round cells count and total count started to increase at D20 ($p=0.07$) to be significantly higher than baseline values at D34, then decreased to baseline values until the end of hyperthermia (D120) with the exception of a low significant value at D73 (Table 1, Fig. 2A).

Sperm chromatin structure assay (SCSA)

Mean (± SEM) sperm DFI (%) was significantly increased at D20 (16.7 ± 3.9), D34 (23.8 ± 2.9) and D45 (31.3 ± 5.4) compared with baseline value (11.9 ± 1.5) (~ 200% increase, Fig. 3B). Mean sperm HDS started to increase as early as D20 (7.4 ± 1.5, $p = 0.07$) and was

significantly higher than baseline value (5.9 ± 0.3) at D34 (10.9 ± 1.0) and D45 (13.0 ± 1.1) respectively (Fig. 3A). The percentage values of sperm DFI (27.8 ± 4.3) and HDS (14.9 ± 2.3, p=0.06) remained higher at D73 but appeared statistically non-significant, most probably due to the smaller number of volunteers (n = 4) at this time point (Fig. 3).

As total sperm count was drastically decreased from D73 until D120 during hyperthermia, the number of spermatozoa was insufficient for SCSA which requires a minimum of 2 million sperm (35). For this reason, both sperm DFI and HDS were analyzed only in one volunteer at D95 and in two at D120 having sufficient number of spermatozoa (Fig. 3).

After Hyperthermia

Sperm motility, viability and count

Percentages of motile and viable spermatozoa, sperm count (Table 1) and total count (Fig 2B) reached respective baseline values at D73 after cessation of hyperthermia.

Round cells

Round cells count and total count did not differ from baseline values except at D73 with a significantly higher value (Table 1, Fig. 2A).

Sperm chromatin structure assay (SCSA)

Percentages values of both sperm DFI and HDS were higher, but statistically non-significant, than baseline values at D45 (n = 3) and recovered respective baseline values from D73 to D180 (Fig. 3).

DISCUSSION

This is the first study in men that reports the drastic but reversible effects of mild induced increase in testicular and epididymal temperature on sperm DNA integrity. To date, an increase in sperm DNA fragmentation in men has been observed in only two case reports (14, 41) of high fever (39-40C°) which correspond to the complex conditions of whole body heating. In order to focus on the testes and epididymides alone, we investigated the effects of a mild increase (+2C°) in testicular and epididymal temperature in men (15 h/day for 120 consecutive days) on sperm parameters and sperm DNA quality.

Based on widely accepted concept of duration of human spermatogenesis, almost 74 days are required for one complete spermatogenic cycle (i.e. spermatogonia to spermatozoa) in men (37). It takes a further ~12 days (1-22) for the spermatozoa to pass through the epididymis and vas deferens and to reach the ejaculate as measured in the only physiological study after incorporation of thymidine-H3 in alive men (42). Therefore, we followed theses authors and the reports on the investigations of the effects of repeated exposures to chemotherapy (38) or radiotherapy (36) on spermatogenesis in men.

Sperm parameters

Effects on epididymal spermatozoa. According to chronology of spermatogenesis and epididymal transit, spermatozoa collected at D4 and D9 during hyperthermia most probably were stored in the epididymis when hyperthermia was induced. At these time points (D4 and D9), we found no change in total sperm count or percentage of motile and viable spermatozoa. This finding is in accordance with previously published animal studies (31, 43).

Effects on testicular germ cells. The spermatozoa collected at D20 were at the elongated spermatids stage (late spermiogenesis) in the testes, and those collected at D34 were at the

late spermatocytes stage (end of meiosis) or early round spermatids stage (beginning of spermiogenesis) when hyperthermia was induced. A significant drop in the percentage of motile spermatozoa occurred as early as D20 during hyperthermia. This fall may be the consequence of two cumulative effects of heating. First, elongated spermatids may be altered during spermiogenesis, as observed 48 h after induced cryptorchidism in rats (44). Second, when passing through the epididymis spermatozoa acquire the capacity of motility, which can be impaired due to inappropriate ion and protein exchange during epididymal transit (45-47). Testicular and epididymal heat stress alters the normal functioning of the epididymis, which may lead to faster sperm epididymal transit, thus reducing the time required for spermatozoa maturation and resulting in a large number of immotile spermatozoa in the ejaculate. This has been reviewed elsewhere in various animal species (7, 8).

The major decrease in sperm output at D34 (~15% of baseline value) during hyperthermia suggests that a 2 °C increase in testicular temperature has an active impact mainly on the meiosis stage. This fall may result from: 1) an early germ cells release from germinal epithelium that was reflected in an increased total round cell count as early as D20 during hyperthermia, and that corresponded to degeneration of germ cells (spermatocytes) resulting from either a direct effect of heat on germ cells, Sertoli-Sertoli and Sertoli-germ cell junctions, and/or on permeability of the blood-testes barrier (48); 2) a possible reduction in cell proliferation and cell survival together with a simultaneous increase in germ cell apoptosis (49) as shown in men after local heating (43 C° in a water bath for 30 minutes for 6 consecutive days). Damage to the seminiferous epithelium 2 weeks after the end of this heat treatment and impaired expression of several proteins mainly expressed during germ cell proliferation, survival and also in apoptosis, was observed (49). Yet, ejaculates at D34 contained spermatozoa (~15% of control value), which suggests that some, but not all, germ cells were more specifically affected by heat; this was reflected in the increased sperm DFI at

D20 during hyperthermia without a drop in sperm output. Moreover, some germ cells could have arrested at the spermatocytes stage. This possibility is supported by the total sperm count values, which started to increase as early as D45 after cessation of hyperthermia. An explanation could be that the sperm ejaculated at D45 were in fact at D33 toward the end of their spermatogenic cycle (45-12 days of epididymal transit = 33 days), and after cessation of hyperthermia spermatocytes restarted their evolutionary process from the spermatocytes stage onward. When heating was withdrawn, arrested spermatocytes were able to regain their differentiation into round spermatids and then into spermatozoa.

Besides, during heating the first stage of spermatogenesis (mitosis and differentiation of spermatogonia) seems not to be affected, as has been reported in induced cryptorchidism in rats (50) and after unilateral cryptorchidism in rabbits (51).

We hypothesize that at the spermatocytes stage some cells underwent apoptosis, some appeared as round cells, a few continued to develop into spermatozoa while others became arrested in a "frozen state". As spermatogonia continued dividing and differentiating, several waves of germ cells accumulated as late spermatogonia B and spermatocytes in the "frozen state"; finally, when heating was stopped, all arrested germ cells restarted their evolutionary process together, giving a sperm output similar to baseline value at D73 after hyperthermia. This concept is further supported by the studies in rodents where scrotal hyperthermia (43°C for 15 minutes) (52) and vitamin A withdrawal (53) resulted in spermatogenesis arrest at spermatogonia in mice, whereas at preleptotene spermatocytes in rats (54).

Sperm DNA fragmentation

Effects on epididymal spermatozoa. No significant change was revealed in percentage of sperm DFI and HDS in spermatozoa collected at D4 and D9 during hyperthermia. Nevertheless, the effects of hyperthermia on the quality of epididymal spermatozoa can not be

neglected. Indeed, the effects of hyperthermia on epididymal spermatozoa depend upon the degree, as observed in mice (34) and duration, as observed in rams (31), of heat exposure. In mice no changes in sperm DNA were reported at 38 °C compared with 40 and 42 °C (30 minutes exposure) while higher embryo loss occurred in rams as early as D4 (epididymal sperm) after a heat exposure of 16h/day for 21 days achieving an intrascrotal temperature ~ 35 °C.

Effects on testicular germ cells. A significant increase in percentage of sperm DFI at D20, D34, D45 and of HDS at D34 and D45 was observed and appeared to continue until the end of the hyperthermia period (D73-D120), but due to the small number of volunteers at D73-D120 these data were not significant. This growing increase in sperm DFI/HDS during hyperthermia may be explained by the chronology of spermatogenesis: (a) at D20, hyperthermia affected the last phase of spermiogenesis (elongated spermatids), when nuclear compaction takes place and the majority of nuclear proteins (histones) are replaced with protamines. During transit through the caput and corpus epididymis when disulfide cross-linking process takes place as shown (55) and reviewed elsewhere (56, 57) sperm were more sensitive to external stresses; (b) at D34, heat affected the cells that were at late spermatocytes and/or early spermatids stages. They travelled the whole spermiogenesis and epididymal transit period thus were exposed to the heat longer than the cells at D20; (c) at D45, the cells were at primary spermatocytes stages and travelled the rest of meiotic stage, whole spermiogenesis and epididymis and were exposed to heat longer than both D20 and D34. This marked increase in sperm DFI and HDS at D34 and D45 is most probably because at these stages (spermatocytes and/or early spermatids) DNA strands are not firmly bound and are less dense than in late spermatids (D20) which is in accordance with the study of Paul et al. (34) in mice where most affected cells were the spermatocytes and spermatids after scrotal heating.

In conclusion longer the time of heat exposure more damaging effects on the DNA quality of ejaculated spermatozoa.

Moreover, increased percentage of DFI, HDS and ratio of histones to protamines after an episode of fever have been shown in human sperm (35). Protamines are critical for proper sperm DNA packaging (58) and may serve a protective function against DNA damage (59). In mice it has been established that protamine haplo-insufficiency is a direct causative factor involved in DNA damage induction (60). Furthermore, DNA polymerase β, which is maximally involved in DNA repair and function during meiosis, lost its activity in rats after artificial cryptorchidism (61).

It is noteworthy that we observed alterations in sperm chromatin integrity when the sperm count was still compatible with natural conception. Damaged sperm DNA may have a negative impact on the fate of the embryo in both natural and assisted reproduction. Some authors have reported reduced blastulation rates after in vitro fertilization (62) and increased frequency of miscarriages (63-65) in women whose partners had high sperm DNA damage compared with women whose partners had low damage. Besides, in rams embryo loss has been reported without any impact on fertilization after scrotal heating (~ 35 °C) (31). Further, impaired embryo development was observed in mice at an ambient temperature of 36 °C (32), or scrotal heating at 40 and 42 °C (34). This shows that sperm with compromised DNA quality can fertilize the oovocyte but a negative impact becomes evident at later stages of embryo development.

Taken together, our findings argue that in conditions of moderate induced testicular and epididymal heat stress (+ 2 °C) sperm chromatin is largely impaired, before the sperm count drops, and recovers when the stress is withdrawn, as has been observed in infertile men with varicocele (66, 67). This leads us to suggest that when increased sperm DNA fragmentation is observed in couples seeking to conceive by assisted reproductive techniques

(ART), underlying cause(s) such as varicocele or occupational habits must first be investigated and treated before moving on to assisted reproductive options. Additionally, it would be useful to evaluate sperm chromatin structure in couples where use of ART has resulted in reduced embryo development and high abortion rates without involvement of a female factor.

To conclude, we present the first study in men that investigated the direct relationship between sperm DNA damage and mild induced testicular and epididymal hyperthermia. We suggest that sperm chromatin may be the earliest indicator of alterations in sperm characteristics in a stress situation. Though additional studies on a larger population are required, our findings may have clinical implications for male contraception specifically during the inhibition and/or recovery phases of spermatogenesis.

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Figure Legends

Figure 1

Chronology of semen sampling. **(A)** Schematic representation of semen sampling timing during the three study periods, before, during and after mild induced testicular and epididymal hyperthermia in men. * represents the days when volunteers underwent complete clinical evaluations during the three study periods. **(B)** represents the location and evolutionary stages of sperm during the spermatogenic process at induction of hyperthermia (D0) and their expected appearance in ejaculates. The bars represent E epididymal sperm, T spermatids, C2 spermatocytes II, C1 spermatocytes 1, G spermatogonia, SC stem cells (modified from May et al., 2000).

Figure 2

Total sperm and round cell counts ($x10^6$/ejaculate) measured during the three study periods, before, during and after mild induced testicular and epididymal hyperthermia in men. **(A)** shows total round cell count and **(B)** total sperm count ($x10^6$). In **(A)** and **(B)** means ± SEM (6 ± 0.9 and 315.24 ± 19.24, respectively) of the three means ± SEM before hyperthermia were compared with the means ± SEM of each data point during and after hyperthermia. However, for easier interpretation of the data all three means before hyperthermia are plotted in the figure. *$p<0.05$. **(C)** represents the location and evolutionary stages of sperm during the spermatogenic process at induction of hyperthermia (D0) and their expected appearance in ejaculates. The bars represent E epididymal sperm, T spermatids, C2 spermatocytes II, C1 spermatocytes 1, G spermatogonia, SC stem cells.

Figure 3

Analysis of sperm high DNA stainability (HDS) and DNA fragmentation index (DFI) by SCSA during the three study periods, before, during and after mild induced testicular and epididymal hyperthermia in men. In **(A)** and **(B)** at all points (•) means ± SEM of all five volunteers are presented except $^{\Psi}n = 4$ and $^{¤}n = 3$, and Arabic numbers (1 and 2) between Ψ and ¤ correspond to the number of volunteers and their mean values who had the minimal required sperm count for SCSA to be performed on the corresponding days. In both **(A)** and **(B),** means ± SEM (5.87 ± 0.3 and 11.9 ± 1.5, respectively, of the three means ± SEM before hyperthermia were compared with the means ± SEM of each data point during and after hyperthermia. However, for easier interpretation of the data all three means before hyperthermia are plotted in the figure *$p<0.05$. **(C)** represents the location and evolutionary stage of sperm during the spermatogenic process at induction of hyperthermia (D0) and their expected appearance in ejaculates. The bars represent E epididymal sperm, T spermatids, C2 spermatocytes II, C1 spermatocytes I, G spermatogonia, SC stem cells.

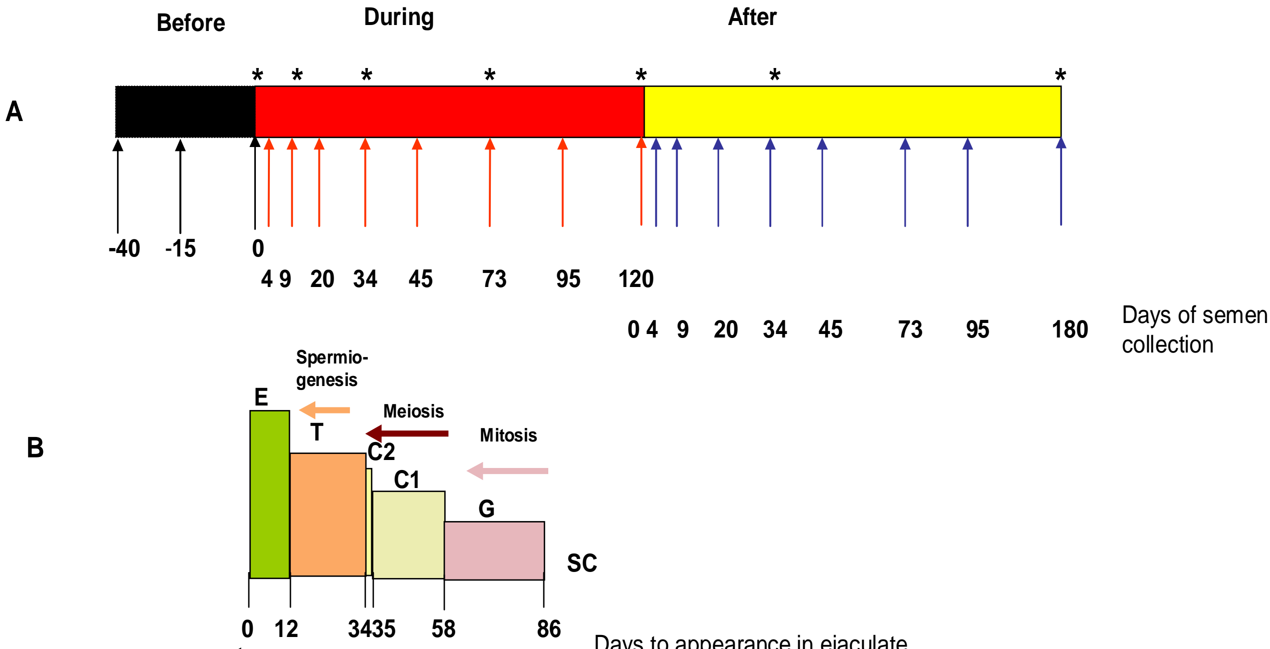


Figure 1

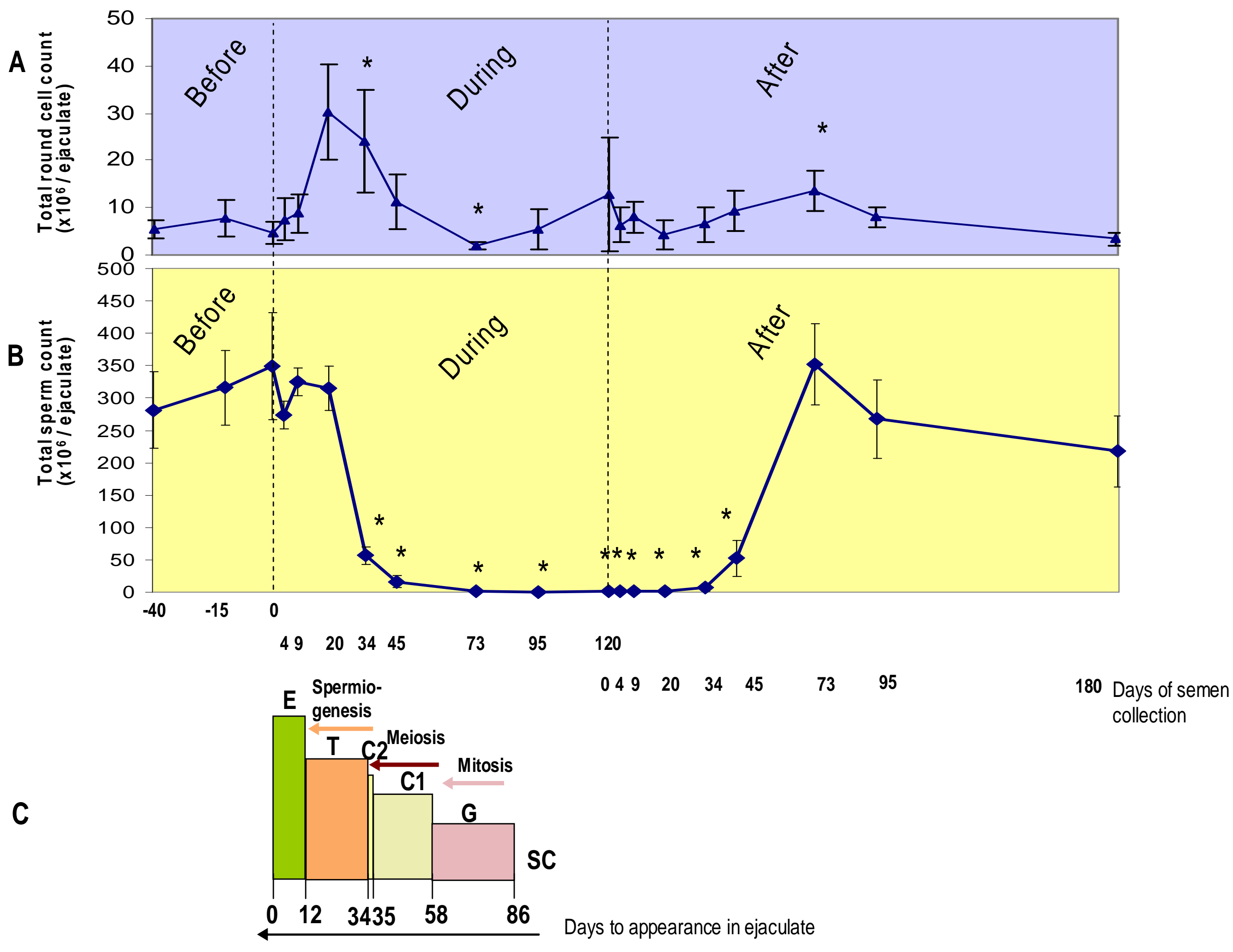


Figure 2

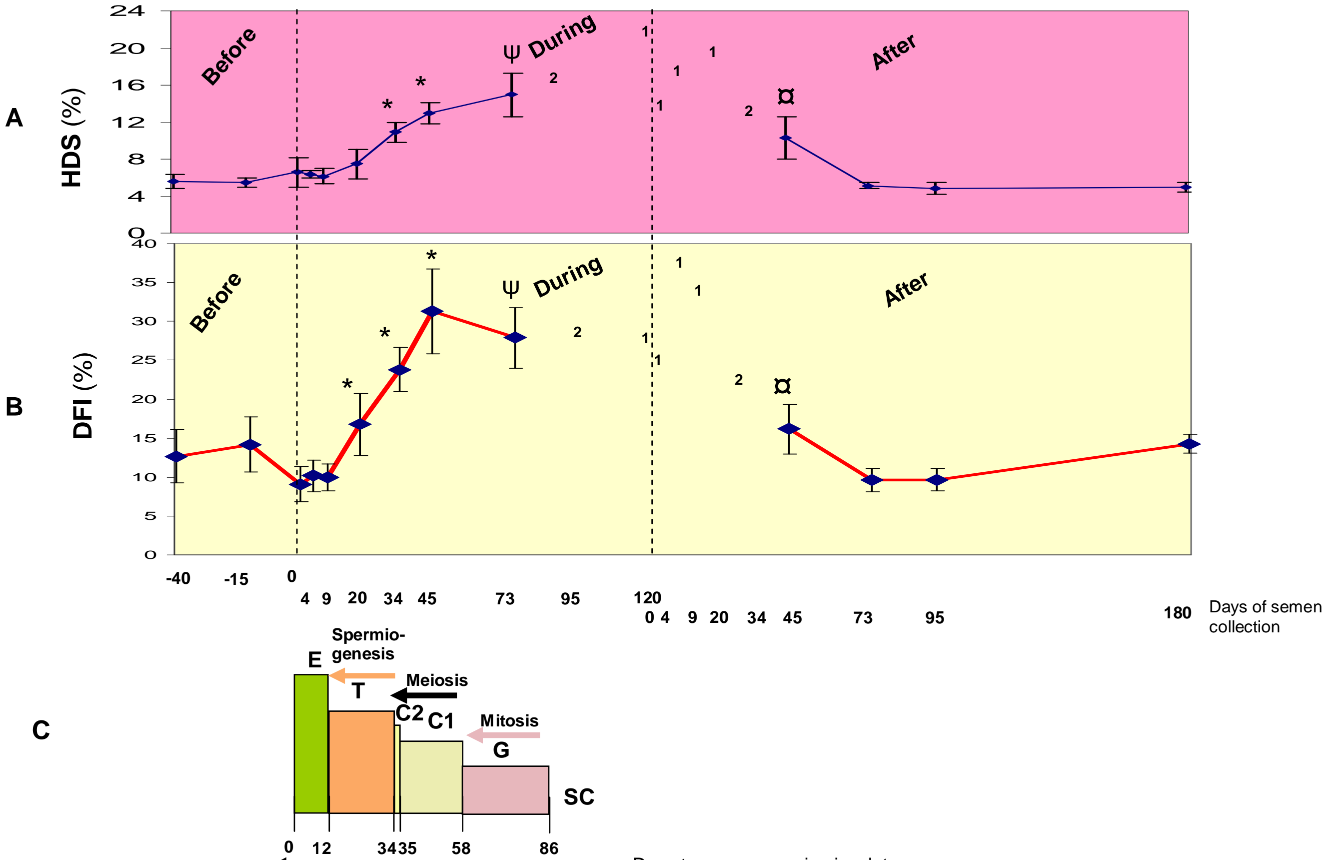


Figure 3

	Hyperthermia																		
	Before			During								After							
Days of semen collection	40	15	0	4	9	20	34	45	73	95•	120	4	9	20	34	45	73	95	180
AD (days)	3.8±0.2	4.4±0.6	4.2±0.2	3.8±0.2	4.2±0.2	4.4±0.2	4±0.3	3.8±0.3	3.8±0.2	4.4±0.4	3.8±0.2	3.8±0.2	4.4±0.2	3.6±0.2	3.6±0.2	3.8±0.3	4±0.0	4.2±0.2	3.8±0.2
Volume (ml)	5.1±1.2	4.1±0.7	5.1±1.4	5.6±1	4.4±0.6	5.0±0.8	4.0±1.1	4.9±1.3	4.2±0.7	4.7±0.7	4.0±0.5	4.1±0.5	5.1±0.9	4.1±0.6	4.1±0.8	4.3±1.0	5.3±1.0	4.5±0.8	4.1±0.6
Sperm count ($\times 10^6$/ml)	62±14.2	83±19.3	88±28.6	68±15.2	75±12.8	68±12.9	15±3.1*	2.8±1.0*	0.4±0.2*	0.04±0.03*	0.4±0.4*	0.2±0.2*	0.2±0.1*	0.2±0.2*	2.2±1.2*	19±11.2*	86±23.1	70±17.1	59.2±19.2
Round cell count ($\times 10^6$/ml)	1.1±0.4	2.0±1.1	1.2±0.6	1±0.5	2±0.8	6.3±1.7	6.0±1.9*	2.1±0.8	0.5±0.8*	0.8±0.5	3.2±2.9	1.6±1.1	1.5±0.6	1.4±1.1	1.7±1.0	2±0.6	3±1.6*	1.9±0.4	0.9±0.3
Sperm viability (%)	70.4±7	72.8±4.6	76.4±2.3	71±3.8	76±3.4	70±3.7	49±9.2*	36±10.4*	23±14.3*	^{a}20±15*	^{b}12.5	13.8^{b}	16^{b}	26±16^{a}	46±19.2^{c}	56±14.3^{d}	75±4.2	77±4.8	72±5.2
Progressive motility (%)	49±5.7	46±2.9	46±3.6	50±5.4	45±4.1	31±2.9*	21±4.8*	^{d}7.4±3.5*	^{a}8.6±7.8*	^{a}7.6±5.8*	^{b}5.0	5.8^{b}	4^{b}	^{c}11±5.0*	^{d}22.2±8*	34±5.3*	41±4.8	49±7.8	45±5

Table 1

Semen parameters before, during and after mild induced testicular and epididymal hyperthermia in men. * = $p<0.05$, AD = abstinence duration.

Values shown are mean ± SEM of 5 volunteers at all time points except: • At D95 $n = 4$, [a] $n = 2$, [b] $n = 1$, [c] $n = 3$ and [d] $n = 4$ men had viable spermatozoa. One man became azoospermic at D95 and another at D120 during hyperthermia, and the remaining 3 men had rare motile or non-motile spermatozoa in their ejaculates. The means (± SEM) of the three means before hyperthermia, which were compared with the means during and after hyperthermia, were AD 4.1 ± 0.1 (days), volume 4.8 ± 0.3 (ml), sperm concentration 77.7 ± 8.1 ($\times 10^6$/ml), round cell count 1.43 ± 0.26 ($\times 10^6$/ml), sperm viability 73.2 ± 1.7 (%) and sperm motility 47 ± 1.0 (%).

3.2. Sperm Morphology

3.2.1. Introduction

Sperm morphology refers to the shape and/or form of the sperm cells. This is an important sperm parameter essential for fertilization. The human spermatozoa are made of a head, a mid piece and a principal piece called tail (Fig 14). This particular structural organization of sperm cells results from complex morphogenetic changes taking place during spermiogenesis. These are (a) the formation of acrosome derived from Golgi vesicles, (b) the formation of an axoneme from the distal centriol, surrounded by periaxonemal structures, which together form the flagellum, (c) the migration and development of mitochondria in a helical sheath around the axoneme in the mid piece, (d) the formation of anisotropic sperm head containing a highly condensed nucleus and, (e) the disappearance of the vast majority of the cytoplasm (Auger, 2010).

3.2.1.1. Abnormalities of sperm morphology

Based on the structural parts of spermatozoa the morphological abnormalities are named according to David's classification (David *et al.,* 1975): seven abnormalities of head, three of mid piece and five of principal piece or tail (Fig 15). It is well established that the percentage of normal sperm has prognostic value of fertility both *in vivo* (Jouannet *et al.*, 1988; Eggert-Kruse *et al.*, 1996; Bonde *et al.*, 1998) and *in vitro* (Kruger *et al.*, 1988; Toner *et al.*, 1995)

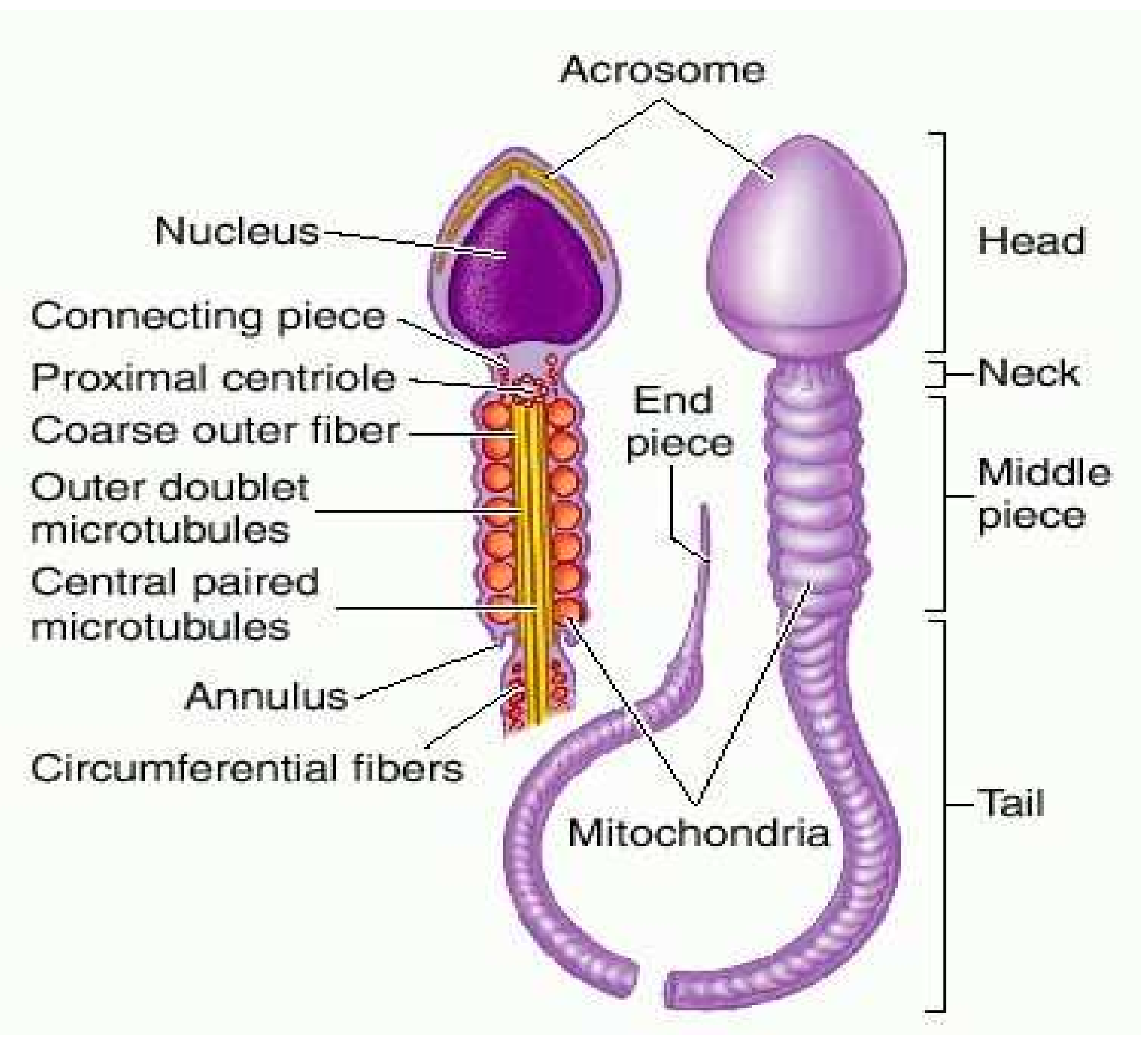
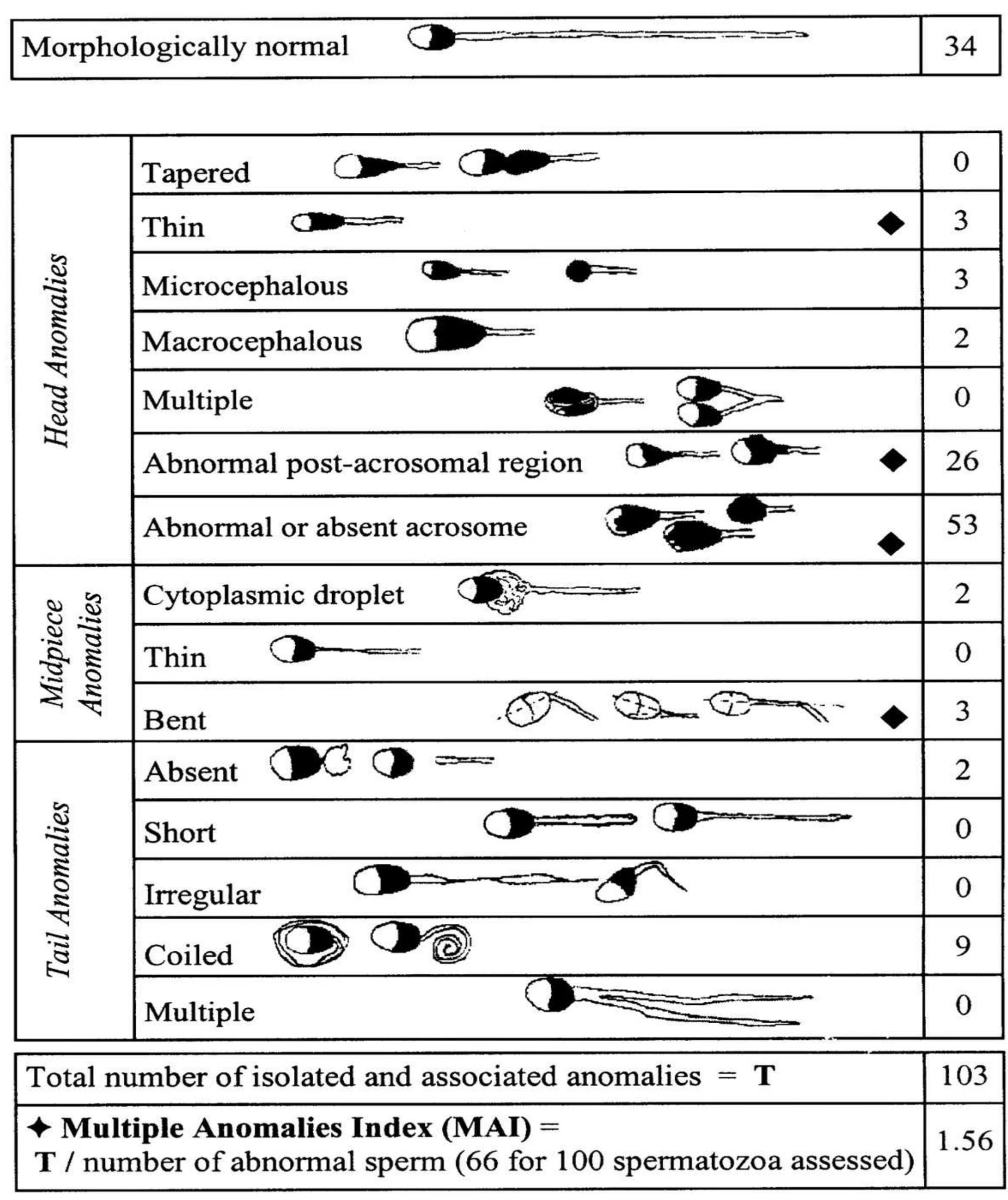


Figure 14. Normal human spermatozoa flat and cross-sectional view (Saunders, 2003).



Morphologically normal		34

Category	Anomaly		Count
Head Anomalies	Tapered		0
	Thin	◆	3
	Microcephalous		3
	Macrocephalous		2
	Multiple		0
	Abnormal post-acrosomal region	◆	26
	Abnormal or absent acrosome	◆	53
Midpiece Anomalies	Cytoplasmic droplet		2
	Thin		0
	Bent	◆	3
Tail Anomalies	Absent		2
	Short		0
	Irregular		0
	Coiled		9
	Multiple		0

Total number of isolated and associated anomalies = **T**	103
✦ **Multiple Anomalies Index (MAI)** = **T** / number of abnormal sperm (66 for 100 spermatozoa assessed)	1.56

Figure 15. Sperm morphology: classifications of normal and abnormal human spermatozoa (David *et al.,* 1975). MAI interpreted by Jouannet *et al*., (1988).

3.2.1.2. Decline in sperm morphology over the period of time and associated factors

Although the precise reason for decline in sperm quality has been of question over the last six decades, several studies have shown a decline in semen quality (Carlsen *et al.*, 1992; Auger *et al.*, 1995; Jensen *et al.*, 1995; Van Waeleghem *et al.*, 1996; Auger, 2010). The main focus of the above studies has been the continuous discussions on decline in sperm count with time. A time related decrease in sperm morphology has been documented in a retrospective Belgian study (Van Waeleghem *et al.*, 1996), in which the authors analysed the sperm characteristics of 416 healthy young men who presented themselves as candidates of sperm donors in the past 19 years. The average proportion of spermatozoa with normal morphology decreased from 39.2 % in the period 1977-1980 to 26.6 % in 1990-1995. In another much larger study, Auger *et al.,* (1995), analysed the sperm characteristics from 1973 to 1992 in 1351 healthy fertile men of Paris region. Linear regression analysis of the age matched close group revealed a decrease in the percentage of normal spermatozoa by 0.7 percent per year. Older age was found to contribute significantly in the decline in the percentage of normal spermatozoa. Similarly a study performed on 214 fertile men reported that age is positively correlated with the increased percentage of microcephalic, macrocephalic and duplicate heads and coiled tail sperm abnormalities (Bujan *et al.*, 1988).

In a more recent study compared to the above cited ones, Auger *et al.,* (2001) reported the sperm morphological defects in relation to medical history, environment and life style in males of pregnant women. From four European centres a total of 1082 men were recruited whom female partner were pregnant by natural conception. Along with life style and medical history, seasonal changes on sperm parameters were taken into account. There were no seasonal variation in normal sperm morphology, however, a number of abnormalities and multiple anomalies index (MAI) was significantly different according to

season especially between spring and winter. The medical, lifestyle and occupational factors modulated significantly the mean percentages of normal spermatozoa. An increase in some sperm abnormalities was associated with medical treatment of mother during pregnancy, higher birth weights and previous treatment of cryptorchidism. Stress, weekly working time, occupational posture and metal welding contributed significantly to several sperm defects (Auger *et al.*, 2001)

3.2.1.3. Testicular hyperthermia and sperm morphology

Effects of scrotal and/or testicular and epididymal hyperthermia on spermatogenesis in men have been investigated over the past fifty years (Procope, 1965; Rock and Robinson, 1965; Robinson *et al.*, 1968; Mieusset *et al.*, 1985; Mieusset *et al.*, 1987a; Shafik, 1992). All these studies have shown a reduction in sperm count and motility. But studies on sperm morphology, an important parameter for fertilization as demonstrated by *in vitro* studies (Rogers *et al.*, 1983; Appleton and Fishel, 1984), in response to a testicular hyperthermia are scarce or produced discrepant results.

There are two reports which highlight the effects of higher testicular temperature on sperm morphology. The first detailed report of sperm morphology after testicular and epididymal hyperthermia using artificial cryptorchidism method was given by Mieusset *et al.,* (1987b). In this study they used the same model as we did in present study and divided the study in three periods; before, during and after hyperthermia. Hyperthermia period lasted for 24 months, while follow up after cessation of hyperthermia was of 18 months. The authors reported a significant increase in head abnormalities in response to testicular hyperthermia. Percentage of elongated heads increased significantly and regularly up to tenth month, thin heads appeared to increase up to the twelfth month and then remained relatively stable

throughout 24 months of hyperthermia period. The percentage of irregular heads increased moderately. Middle piece abnormalities included the percentage increase in bent tails started from fourth month and remained constantly higher till the twenty-fourth month. After cessation of hyperthermia mean percentage of abnormally shaped spermatozoa remained significantly higher than baseline values up to sixth months after heat treatment.

In another more recent study the effects of febrile illness on sperm quality variation were reported (Carlsen *et al.*, 2003). Twenty-seven healthy men were followed up for a history of febrile illness for up to sixteen months. Monthly semen samples were collected from all men and daily records of occurrence of experienced febrile episode was noted (without temperature measurements). A significant increase in the percentage of abnormal shaped spermatozoa was observed when the febrile episode occurred at the time when ejaculated spermatozoa were going through spermiogenesis (post meiotic) (Carlsen *et al.*, 2003). In an other study performed 60 years ago (MacLeod, 1951), the author followed three medical students during a febrile disease of chickenpox and pneumonia and, found an increase in abnormal shaped spermatozoa which recovered around thirty days after normalization of the temperature.

The objective of analysing sperm morphology in our study was to investigate the effects of a mild testicular and epididymal temperature (lower than physiological body temperature). Although, the above cited studies reported the effects of testicular temperature on sperm morphology. However, in the two studies (MacLeod, 1951; Carlsen *et al.*, 2003) the temperature was supra- physiological which corresponds to a disease or pathological condition (fever) and these studies are different from ours in which only testis and epididymis temperature was increased but was lower than core body temperature. In the study of Mieusset *et al.,* (1987b), though the temperature was similar as achieved in our study but it lacks the data specific to spermatogenesis stages.

We, in the present study, report the spermatogenesis stage specific effects of a mild induced testicular and epididymal hyperthermia on sperm morphology.

3.2.2. Methodology

Semen collection was performed by masturbation and the same procedure of sampling was followed as reported for sperm DNA fragmentation. Semen smears were prepared by putting a drop of 10 μl on a glass slide, air dried and then fixed for at least 1h with a mixture of absolute ethanol (2/3) and acetic acid (1/3). Sperm morphology was assessed by observing at least 100 spermatozoa per sample, studied under an optical microscope after fixation and staining (Papanicolaou modified: haematoxylin Harris, Shorr). The detailed study of the abnormal forms was undertaken according to David *et al.,* (1975). The head showing lysis or distortion were not counted. A minimum of 100 sperm were counted for morphology. According to the classification of David *et al.,* (1975) modified by Jouannet *et al.,* (1988) the sperm abnormalities include:

Head: elongated, thin, micro head, macro head, duplicate/multiple, abnormal or absent acrosomal region, abnormal base

Mid piece: cytoplaspic droplet, thin, bent

Tail: bent, absent, short, coiled, multiple

Multiple anomalies index (MAI):

MAI = Total number of anomalies identified on spermatozoa / number of abnormal spermatozoa

Multiple anomalies index

MAI is considered one of the most predictive factor in fertility assessment (Jouannet *et al.*, 1988). Generally, MAI equal to or less than 1.6 is considered favourable to achieve a pregnancy, however, inter laboratory variations occur worldwide. For example, in a prospective study of 394 infertile men conducted over three years Jouannet *et al.,* (1988) showed decreased pregnancy rates in groups where MAI was greater than 1.6 after 1 or 3

years. In order to avoid the bias of inter laboratory variations the correct norms of the laboratory while assessing the sperm morphology are important. The design of our study is such that it reduces such biasness because morphology was assessed before induction of hyperthermia, during and after the cessation of hyperthermia. Moreover, the analysis was performed by a single reader during all three study periods i.e. before during and after which further minimizes inter individual variations.

3.2.3. Statistics

Means (± SEM) of the three means (± SEM) of the three data points (each data point $n = 5$) before hyperthermia were compared with the means of each data point ($n = 5$) during and after hyperthermia (significance $p<0.05$). Mean values of sperm morphology were compared by applying non-parametric Wilcoxon's test using STATA software version 8.

3.2.4. Results

We observed MAI, head, mid-piece and tail abnormalities/defects, the results of which are described in relevant sections. No difference in semen volume and sexual abstinence delay was observed during the three study periods i.e. before, during and after hyperthermia.

3.2.4.1. Multiple anomalies index (MAI) of sperm morphology

Before hyperthermia

Mean of multiple anomalies index of the three means before hyperthermia (control) which was compared with values of during and after hyperthermia was 1.94±0.02.

During hyperthermia

Mean (±SEM) multiple anomalies index of sperm morphology appeared significantly higher than the value before hyperthermia (control) (1.94±0.02) as early as D9 (2.1±0.05) (i.e. epididymal sperm) and remained significantly higher during the entire period of hyperthermia with exceptions at D20 ($p=0.06$), D95 and D120 where the values were higher than the control but the difference was not significant. The reason for this lacking significance was due to less number of volunteers assessed at these time points. At D20 due to breakage of one slide only four volunteer's samples were examined for sperm morphology. At D95 and D120 only two volunteers had sufficient sperm cells to perform the morphological analysis while the remaining were either azoospermic (n=2) or hade rare spermatozoa (n=1) in the ejaculates (Fig 16).

After hyperthermia

Mean multiple anomalies index (MAI) of sperm morphology was non-significantly higher than control (1.94±0.02) at D4 (2.8±0.2), D9 (3.1±0.3), D20 (2.7±0.01) and D34 (2.29±0.3) and, was significantly higher at D45 (2.3±0.1). The MAI returned to control values at D73 after cessation of hyperthermia and remained at base level until the end of the study period (Fig 16).

The reason of lacking significance at D4, D9, D20 and D34 was due to because at these time points we had only two volunteers with sufficient number of sperm while at D45 all five volunteers recovered enough spermatogenesis and the difference achieved significance.

Conclusions

Under mild testes and epididymal hyperthermia MAI increased significnalty compared with control as early as D9 of hyperthermia, remained higher till D45 after hyperthermia and rocoverd the control value at D73 after the cessation of hyperthermia.

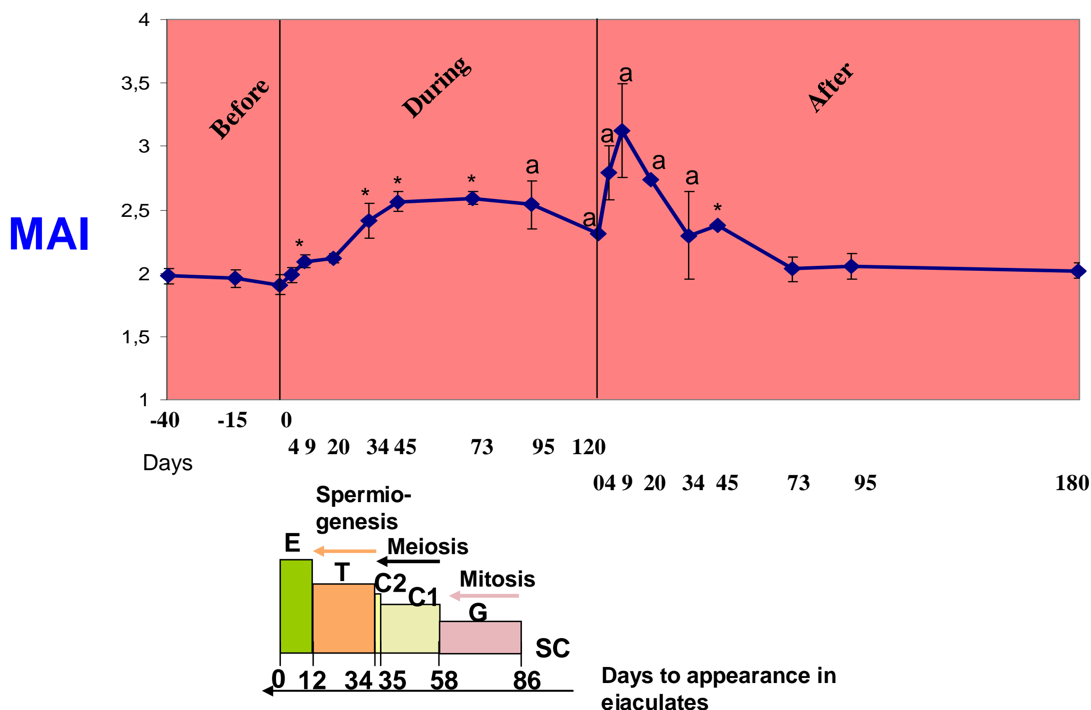


Figure 16. (A) Multiple anomalies index (MAI) of sperm morphology before, during and after mild induced testicular and epididymal hyperthermia in men. Mean ± SEM (1.94±0.02) of the three means ± SEM before hyperthermia was compared with the means ± SEM of each data point during and after hyperthermia. However, for easier interpretation of the data all three means before hyperthermia are plotted in the figure. *$p<0.05$, [a]n=2**.** **(B)** Represents the location and evolutionary stages of sperm during the spermatogenic process at induction of hyperthermia (D0) and their expected appearance in ejaculates. The bars represent E epididymal sperm, T spermatids, C2 spermatocytes II, C1 spermatocytes I, G spermatogonia, SC stem cells.

3.2.4.2. Head defects

Results of the abnormalities of sperm head are presented in Table 4.

Before hyperthermia

The means of three means before hyperthermia (control) which were compared for statistical analysis are: elongated (0.93±0.6), thin (7.6±0.2), micro head (18±0.9), macro head (1.0±0.4), multiple heads (0.8±0.2), deformed head base (23±0.4) and acrosome defects (43.6±0.6). However, for easier interpretation of the data all three means before hyperthermia are given in the Table 4.

During hyperthermia

Elongated

The mean percentage of elongated sperm head was not significantly different than control during the entire hyperthermia period.

Thin

The mean percentage of thin head spermatozoa increased significantly compared with control at D34, D45 and D73 during hyperthermia. However, at D95 and D120 no significant difference was revealed.

Micro-head

The mean percentage of micro head spermatozoa was significantly higher than control at D34 and was discernibly higher but non-significant at D45, D73 ($p=0.07$) and at D95 and D120.

Macro-head

No difference in percentage of macro head spermatozoa was observed compared with control.

Multiple heads

The mean percentage of multiple head spermatozoa although appearing higher than control starting from D34 till the end of hyperthermia period, revealed significance only at D73. The lacking significance most probably was due to greater variations among the means.

Deformed head base

The mean percentage of spermatozoa with abnormal base of the head started to increase, compared with control, at D34 and reached significant level at D73 and remained elevated till the end of hyperthermia period.

Acrosome defects

The mean percentage of spermatozoa with defective acrosome started to increase as early as D20 ($p=0.06$) and remained significant at D34, D45 and D73 and was discernibly higher but non-significant than control at D95 and D120.

At D95 and D120 during hyperthermia the maximum number of volunteers assessed for sperm morphology was two which rendered the difference non-significant at these data points.

After hyperthermia

Elongated

The mean percentage of spermatozoa with elongated head was not significantly different than control except at D45 after cessation of hyperthermia.

Thin

The mean percentage of spermatozoa with thin head was not significantly different than control except at D45 after cessation of hyperthermia.

Micro head

No significant difference was observed in the percentage of spermatozoa with micro heads after cessation of hyperthermia.

Macro head

No significant difference was observed in the percentage of spermatozoa with macro heads after cessation of hyperthermia.

Multiple heads

Higher but non-significant difference was observed in the percentage of spermatozoa with multiple heads after cessation of hyperthermia from D9 till D45 which reached control value at D73 till the end of follow up period.

Deformed head base

The mean percentage of spermatozoa with deformed head base was not significantly different from the control except at D45 after cessation of hyperthermia.

Acrosome defects

The mean percentage of spermatozoa with deformed or absent acrosome was non-significantly higher than control from D4 till D45 after cessation of hyperthermia and reached control value at D73 till the end of follow up period.

From D4 till D34 after cessation of hyperthermia the maximum number of volunteers assessed for sperm morphology was two which rendered the difference non-significant at these data points.

Conclusions

Under mild testes and epididymal hyperthermia, the earlier increase in head abnormalities was seen as early as D20 of hyperthermia and remained higher (most of the head defects) till D45 after hyperthermia. The recovery of head defects started to occur after D45 after cessation of hyperthermia and reached respective control values at D73 after the cessation of hyperthermia.

Table 4. Percentage of sperm head anomalies before, during and after mild induced testicular and epididymal hyperthermia in men.

	Hyperthermia																		
	Before			During								After							
Days of semen collection	40	15	0	4	9	20	34	45	73	95	120	4	9	20	34	45	73	95	180
Elongated	1±0.7	0.8±0.3	1±0.8	0	0.6±0.2	0.3±0.2	0.4±0.4	1±1	3±1	[a] 2.5±1.5	[a] 0	[a] 0	[a] 2±2	[a] 4±1.5	[a] 1±1	5±2*	1±0.4	0.8±0.3	0.4±0.2
Thin head	7±1	8±3	8±2	7±3	10±3	6±2	24±7.3*	20±4*	18±4*	[a] 20±6	[a] 7±5	[a] 15±13	[a] 6±6	[a] 24±6	[a] 18±15	17±4*	12±3	8±2	7±2
Micro-head	20±4.5	19±3	17±3	19±4	16±2	23±3	29±4*	30±7.5	34±7	[a] 29±3.5	[b] 36	[b] 32	[b] 24	[a] 31±1	[a] 24±7	17±5	18±3	18±4	16±3.5
Macro-head	0	1±0.4	2±0.8	0.6±0.4	0.6±0.4	1±0.4	0.4±0.2	1±0.6	0.6±0.4	[a] 2±2	[a] 2±2	[a] 3±3	[a] 2±2	[a] 1±1	[a] 0	3±0.9	1±0.7	0.2±0.2	1±0.3
Multiple heads	1±0.7	0.4±0.2	1±0.5	1±0.5	3±1	1±0.5	3±1.5	4±1	3±0.8*	[a] 4.5±0.5	[a] 2.5±15	[b] 2	[b] 4	[a] 6±0.5	[a] 8±4.5	3±2	2±2	2±2	2±1
Deformed head base	23±5.5	23±6	22±3.5	29±7	29±7	20±2	28±6	36±10	34±8*	[a] 29±6	[b] 25	[b] 12	[b] 52	[a] 26±4	[a] 28±1	43±8*	26±4	23±5	23±6
Acrosome defects	44±3	44±5	43±3	50±3	48±2	53,5±3	77±8*	85±5*	85±4*	[a] 88±6.5	[a] 87±4.5	[b] 92	[b] 90	[a] 99±1	[a] 77±16	67±6.5	47±4	47±5	47±6

Means of the three means before hyperthermia which were compared for statistical analysis are: elongated (0.93±0.6), thin (7.6±0.2), micro head (18±0.9), macro head (1.0±0.4), multiple heads (0.8±0.2), deformed head base (23±0.4) and acrosome defects (43.6±0.6). However, for easier interpretation of the data all three means before hyperthermia are given in the table.

*n = $p < 0.05$, [a]n = 2, [b]n = 1

3.2.4.3. Mid piece defects

Results of the abnormalities of mid piece are presented in Table 5.

Before hyperthermia

Means of the three means before hyperthermia which were compared for statistical analysis are: cytoplasmic droplet (4.3±0.4), thin (1.6±0.2) and bent (18.6±0.8).

During hyperthermia

Cytoplasmic droplet

The percentage of spermatozoa with cytoplasmic droplet was non-significantly higher than control.

Thin

The percentage of spermatozoa with thin mid piece was non-significantly higher than control.

Bent

The percentage of spermatozoa with bent mid piece started to increase at D34 ($p=0.07$) and was significantly different than control at D45. The values were also higher than control at D73 and D95 compared but the difference was not statistically significant.

After hyperthermia

Cytoplasmic droplet

The percentage of spermatozoa with cytoplasmic droplet was not significantly different from the control after cessation of hyperthermia.

Thin

The percentage of spermatozoa with thin mid piece was not significantly different from the control.

Bent

From D4 till D34 after cessation of hyperthermia the percentage of spermatozoa with bent mid piece was non-significantly higher than control and was significantly different at D45 and, reached control value at D73 till the end of follow up period.

Conclusions

Abnormalities of mid-piece appeared higher but non-significant as compared to control during hyperthermia till D45 after hyperthermia and reached respective control values at D73 after cessation of hyperthermia.

Table 5. Percentage of sperm mid piece anomalies before, during and after a mild induced testicular and epididymal hyperthermia in men.

	Hyperthermia																		
	Before			During								After							
Days of semen collection	40	15	0	4	9	20	34	45	73	95	120	4	9	20	34	45	73	95	180
Cytoplasmic droplet	4±1.6	4±1.2	5±1.2	5±1.7	6±2.2	2±0.8	5±1	4±0.8	3±1.4	[a]6±2	[a]2±1.5	[b]0	[b]0	[a]12±2	[a]7±1	9±2.2	5±0.5	5±0.6	4±1.6
Thin	2±0.9	2±1	1±1	4±3	3±1	3±1.5	2±2	3±1	2±0.7	[a]1±0.5	[a]1±1	[b]0	[b]4	[a]2±1.5	[a]4±1	4±2.3	3±1.7	3±2.2	3±1.7
Bent	20±2	17±3	19±3.5	18±4	20±4	17±5	28±4	27±2*	24±1	[a]25±7	[a]17±8	[b]40	[b]26	[a]2 9±3.5	[a]17±2	24±2*	22±3	21±3	18±3

Means of the three means before hyperthermia which were compared for statistical analysis are: cytoplasmic droplet (4.3±0.4), thin (1.6±0.2) and bent (18.6±0.8). However, for easier interpretation of the data all three means before hyperthermia are given in the table.

*n = $p < 0.05$, [a]n = 2, [b]n = 1

3.2.4.4. Tail defects

Results of the abnormalities of sperm tail are presented in Table 6.

Before hyperthermia

Means of the three means before hyperthermia which were compared for statistical analysis are: absent (1.3±0.3), short (1.3±0.2), irregular (1.3±0.3), coiled (12.6±1.6) and multiple (1.0±0.06).

During hyperthermia

Absent

The mean percentage of spermatozoa with missing/absent flagella was significantly higher than control at D34 and D45 and remained non-significantly higher at D73 and D95.

Short

No difference in the percentage of spermatozoa with short tail was observed during the entire hyperthermia period.

Coiled

The mean percentage of spermatozoa with coiled tail was significantly higher than the control as early as D34 and remained higher during the entire period of hyperthermia. However, the difference was not significant at D95 and D120.

Irregular

No difference in the percentage of spermatozoa with irregular tail was observed during the entire hyperthermia period.

Multiple

The mean percentage of the spermatozoa with multiple flagella was significantly higher than control as early as D34 and remained higher during the entire period of hyperthermia except at D120.

After hyperthermia

Absent

No significant difference in the percentage of spermatozoa with missing/absent flagella was observed after the cessation of hyperthermia.

Short

No difference in the percentage of spermatozoa with short tail was observed.

Coiled

From D4 till D34 after cessation of hyperthermia the percentage of spermatozoa with coiled tail was non-significantly higher compared with control but was statistically significant at D45 which returned to control value at the end of follow up period.

Irregular

No difference in the percentage of spermatozoa with irregular tail was observed.

Multiple

No significant difference in the percentage of spermatozoa with multiple flagella/tail was observed after the cessation of the hyperthermia.

Conclusions

An increase in sperm tail abnormalities compared to controls was observed as early as D34 of hyperthermia which remained higher throughout the entire period of hyperthermia till D45 after hyperthermia. Recovery to the respective controls started from D45 after the cessation of hyperthermia and reached repective control values at D73.

Table 6. Percentage of sperm tail anomalies before, during and after a mild induced testicular and epididymal hyperthermia in men.

	Hyperthermia																		
	Before			During								After							
Days of semen collection	40	15	0	4	9	20	34	45	73	95	120	4	9	20	34	45	73	95	180
Absent	1±0.6	1±0.4	2±0.6	2±0.7	1±0.7	1±0.9	3±0.7*	5±1*	5±2.7	^{a}3±1	^{a}1±1	^{a}0	^{a}2±2	^{a}2±2	^{a}6±1.5	2±0.5	1±0.5	1±0.3	3±1
Short	1±0.2	1±0.3	2±0.9	1±0.4	1±0.3	1±0.5	3±1	4±2	3±0.7	^{a}4±1	^{a}2±1.5	^{b}1	^{b}1	^{a}8±2.5	^{a}2±1.5	2±0	1±0.3	1±0.9	1±0.3
Irregular	1±0.7	2±1	1±0.3	2±0.8	2±1	1±0.3	3±0.9	3±1	1±0.5	^{a}1±1	^{a}2±1.5	^{b}4	^{b}2	^{a}6±4	^{a}2±2	2±0.9	2±1	2±1	1±0.2
Coiled	13±3	15±2	10±3	10±1.5	14±2.5	14±4	22±3.5*	27±3	20±5	^{a}30±2	^{a}24±3.5	^{b}20	^{b}26	^{a}22±3.5	^{a}14±4.5	14±1*	13±2	13±3	12±3
Multiple	1±0.5	1±0.3	1±0.3	1±0.7	3±0.8	1±0.2	2±0.5*	2±0.6*	3±0.6*	^{a}2±0	^{a}1±1	^{a}0	^{a}1±1	^{a}3±3	^{a}4±2.5	4±1	2±0.5	1±0.2	2±0.4

Means of the three means before hyperthermia which were compared for statistical analysis are: absent (1.3±0.3), short (1.3±0.2), irregular (1.3±0.3), coiled (12.6±1.6) and multiple (1.0±0.06). However, for easier interpretation of the data all three means before hyperthermia are given in the table.

*n = $p < 0.05$, [a]n = 2, [b]n = 1

3.2.5. Discussion

MAI has been reported to be one of the most predictive value in fertility assessment of couples (Jouannet *et al.*, 1988). Ours is the first study that shows a significant increase in mean MAI as early as D9 after the onset of a diurnal artificial cryptorchidism (DAC). Such an increase remained persistent during the entire period of hyperthermia till D45 after hyperthermia and returned to control value at D73 after the cessation of hyperthermia.

It is difficult to compare the results of changes in sperm morphology of different hyperthermia experiments because of variations in the source, intensity and duration of heating as observed in extended periods of local (Rock and Robinson, 1965) and whole body heating (Procope, 1965). However, our results confirm the previously published results using the same model (Mieusset *et al.*, 1987b) in which they extended the DAC period up to 24 months but the results were not interpreted with the chronology of spermatogenesis. Our results, in general, are also in accordance with the results of a more recent study (Carlsen *et al.*, 2003) in which the authors followed 27 healthy volunteers for up to 16 months with occurrence of a febrile episode. They argued that fever causes an increase in the percentage of abnormal shaped spermatozoa particularly if the febrile episode occurred when the ejaculated sperm were going through spermiogenesis (post meiotic). In accordance with Carlsen *et al.,* we also observed a significant change in MAI and individual sperm morphology defects during the period of spermiogenesis (D20, D34). Additionally, we observed a significant increase in MAI at D9 during hyperthermia the time when ejaculated spermatozoa were already stored in the epididymis when hyperthermia was induced. We do not know the mechanism how mild heat induction caused morphological changes in epididyaml spermatozoa. The one possible reason might be the altered epididymal functions under heat stress (Bedford, 1991, 1994) which may lead to unbalanced ion exchange or modifications in the physio-chemical properties of

epithelial cells. These modifications may impair the incorporation of necessary elements of epididymis required for normal sperm morphology. This increase in MAI and also individual abnormalities became more profound at D34 and D45 which represent the early spermatids, in accordance with Carlsen *et al.*, (2003), and spermatocytes stages (Mieusset *et al.*, 1987b) respectively. Though, Mieusset *et al.,* did not report the spermatogenesis stages but the individual morphological defects were observed during the 2nd month of hyperthermia which corresponds to D45 in our experiments (spermatocytes stage). Studies have shown that the higher the MAI the lower the chances are of pregnancy (Ducot *et al.*, 1988; Jouannet *et al.*, 1988).

Our model is different from that of the febrile illness approach because in our case the testicular and epididymal temperature was always less than core body temperature while in case of fever the body temperature ranged from 39-40 °C corresponding to a pathological condition.

Three important factors which can affect semen characteristics and are liable to induce bias in semen studies are: abstinence period, individual variability and seasonal influence. Abstinence has been found to affect all semen characteristics except the sperm morphology (Schwartz *et al.*, 1979; Poland *et al.*, 1985). Further, in our study the abstinence delay was constant thus variations in the percentage of abnormal spermatozoa were not influenced by this factor. Individual variations also existed in our results which at certain data points rendered the difference statistically non-significant. Therefore, to minimise the bias, we took the mean of the three means before hyperthermia (control) and compared with the means of each data point during and after hyperthermia. Further, seasonal influence has been found to be associated with changes in sperm morphology according to some authors (Spira, 1984; Auger *et al.*, 2001) while others found no seasonal correlation with sperm morphology (Ombelet *et al.*, 1996; Centola and Eberly, 1999). Although we did not start

experimentations on all volunteers at once but the difference of time was small i.e. the first volunteer gave semen sample in March and the last in May and continued for a year. Therefore, no seasonal variations could explain the modifications in the percentage of abnormal spermatozoa.

Indeed, the changes in sperm morphology observed in our experiments relate to the effects of mild induced testicular and epididymal hyperthermia. In fact, with the induction of hyperthermia the mean percentage of abnormally shaped spermatozoa and different types of defects started to rise progressively and the major effect was seen at D34 and D45 during hyperthermia where majority of the sperm morphological defects became significantly different than respective controls. The heating method used in our experiments realised an increase of 1-2 °C in the testes and epididymis temperature. Nevertheless, other factors such as scrotal vascularization/blood flow could have been affected by DAC as observed in rams (Fowler and Setchell, 1971) and rats (Glover, 1966; Waites *et al.,* 1973) when scrotal temperature was higher than 34 °C. Additionally, increased flow of testicular blood plasma has been reported during local heating (at 36 °C and 39 °C for 60 minutes) of the testes of rams (Mieusset *et al.*, 1992b).

After cessation of hyperthermia the percentage of normal spermatozoa returned to control values maximum by D73 which is different to the report of Mieusset *et al.,* (1987a) in which the recovery occurred months after cessations of hyperthermia. This could be due to the length of duration of testicular and epididymal heat exposure which in their case was 24 months while it was 4 months in our study. Besides, the recovery in the study of Carlsen *at al.,* (2003) occurred 30 days after the normalization of temperature after an episode of febrile illness.

In conclusion our findings show significant adverse effects of a mild induced testicular and epididymal hyperthermia on sperm morphology not only during the more sensitive stages

of spermatogenesis but also on epididymal spermatozoa. Yet, the mechanisms involved in the modifications of sperm morphology under mild testes and epididymal hyperthermia are not clear; these findings subject to further confirmation may be of significance in further morphological investigations specific to spermatogenesis stages.

3.3. Acid Aniline Blue Study

3.3.1. Introduction

It has been reported that 15% of infertile men have normal sperm parameters (Agarwal and Allamaneni, 2005a), therefore, basic semen analysis seems not sufficient for the evaluation of male fertility status. Several investigating areas in male fertility such as sperm morphology, sperm motility, acrosome reaction, chromatin condensation, aneuploidy and DNA fragmentation, have been a focus of research since a long time. In continuation to the search of improvement in the diagnosis of male infertility, several tests have been developed over the past decade (Lewis, 2007). Of these, the COMET, TUNEL and sperm chromatin structure assay (SCSA) have been used in chromatin/DNA quality evaluation (Agarwal and Said, 2005b; Lewis and Aitken, 2005; Aitken and De Iuliis, 2007; Evenson *et al.*, 2007).

Among the tests used for DNA quality assessment acid aniline blue staining (AAB) (Terquem and Dadoune, 1983) has also been advised by researchers to improve the assessment of fertility in men (Auger *et al.*, 1990; Hammadeh *et al.*, 2001). This method can be considered as an index of sperm quality (Dadoune *et al.*, 1988; Roux and Dadoune, 1989). The nuclear maturity may be implicated in human fertility as a relationship between the electrophoretic pattern of nuclear proteins and the percentage of aniline blue stained sperm heads as reported for infertile men (Chevaillier *et al.*, 1987). For example, in a retrospective study of 49 fertile and 396 suspected infertile men, Auger *et al.,* (1990) stained the semen samples with aniline blue to assess sperm chromatin condensation along with several other semen parameters (sperm motility, concentration, morphology, pH, volume, zinc fructose). Among the 13 semen variables analysed, the aniline blue test (unstained sperm heads) was the fifth variable that was significantly correlated to fertility. The results of aniline blue test were also able to differentiate between donor and patient groups. Besides, in a case control study of 90 patients and 75 controls (Hammadeh *et al.*,

2001), a total of 165 semen samples were examined for chromatin condensation (aniline blue stain) along with other classical sperm parameters (morphology, motility, count). A significant difference among the patients and the controls was found where a greater percentage of unstained sperm heads was observed in control group compared with the patients independent of the other sperm parameters (Hammadeh *et al.*, 2001). Jeulin *et al.,* (1986) studied the correlation between various semen characteristics and the rate of fertilization (IVF) and, reported that out of seven multiple significant correlations five included the aniline blue test results i.e. mature/condensed head. When decondensing properties of sperm heads between the two groups of subfertile patients were compared, a significantly higher percentage of unstained sperm nuclei were observed in normospermic semen samples than semen with lower characteristics (Colleu *et al.*, 1988). Other studies have shown abnormalities of the protein pattern linked to DNA of four infertile patients selected on the basis of sperm with low percentage of mature heads (Chevaillier *et al.*, 1987).

To the best of our knowledge no study has been documented on human sperm chromatin assessment by aniline blue test after exposure of human testes and epididymis to a heat stress. Therefore, the objective of these experiments was to analyse the changes in sperm chromatin maturity in response to a mild induced testicular and epididymal hyperthermia in men.

3.3.2. Materials and methods

3.3.2.1. Acid aniline blue stain

Acid aniline blue (AAB) stain assesses the degree of sperm nuclear condensation or maturation.

Principal

The AAB discriminates between lysine-rich histones on the one hand, and arginine and cysteine-rich protamines on the other hand (Auger *et al.*, 1990). This test provides a specific positive reaction for lysine and reveals the differences in the composition of basic nuclear proteins of ejaculated spermatozoa. The nuclei of immature spermatozoa are rich in histones and contain abundant amount of lysine. When stained with AAB the immature spermatozoa take up the stain and react positively to the test, whereas, the mature spermatozoa have abundant amount of ariginine and cystenine, and appear negative to the test (Hofmann and Hilscher, 1991).

Sample preparation

Semen collection was performed by masturbation and same procedure of sampling was followed as reported for sperm DNA fragmentation and sperm morphology in corresponding sections.

Technique

A volume of 50 µl of raw semen samples was washed with 10 ml of Dulbecco's phosphate buffer saline (DPBS pH 7.4, 1X, GIBCO, Auckland, New Zealand) by centrifugation at 1500g for 10 minutes. The supernatant was removed leaving 20 µl in the tube to mix the

pellet. A drop of 10 µl was smeared on a glass slide. The slides were air-dried and fixed for 30 minutes in 3% glutaraldehyde in DPBS. The slides were washed twice for 10 minutes each in distil water. The smears were dried and stained for 5 minutes in 5 % aqueous aniline blue solution (pH 3.5). Slides were rinsed in distil water and air-dried. A minimum of 100 spermatozoa per slide were counted under bright field microscope except when sperm count was very low and rare spermatozoa were found on the slide. Sperm heads which contained immature nuclear chromatin took up the stain and appeared blue and those with mature nuclear chromatin did not take the stain and appeared white or transparent (Fig 17).

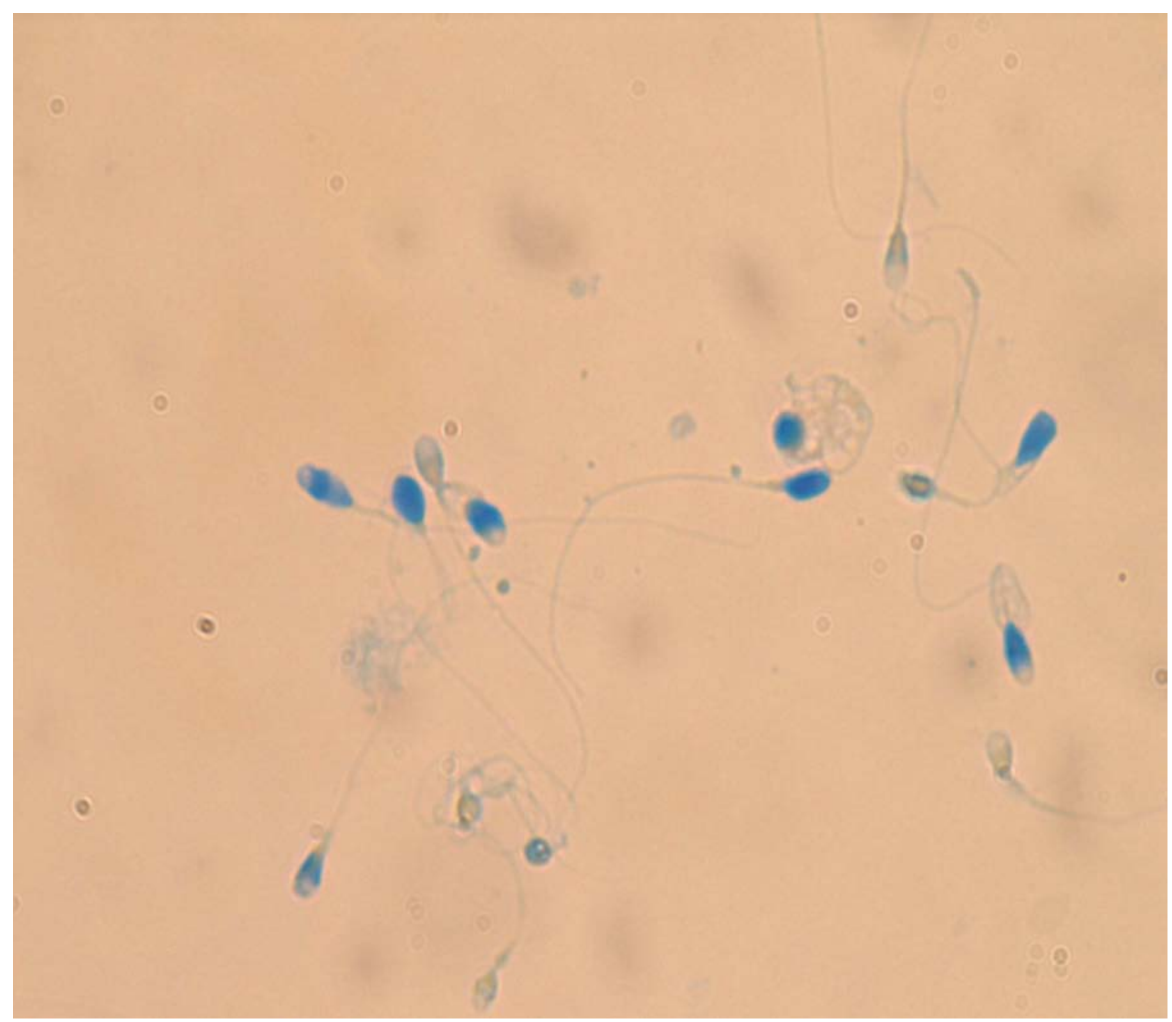


Figure 17. Aniline blue staining: sperm with blue heads are aniline blue positive and with transparent heads are negative.

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3.3.3. Statistics

Mean (± SEM) of the two means (± SEM) of the two data points (each data point $n = 5$) before hyperthermia were compared with the means of each data point ($n = 5$) during and after hyperthermia (significance $p<0.05$). Mean percentage values of aniline blue positive spermatozoa (stained heads) were compared by applying non-parametric Wilcoxon's test using STATA software version 8.

3.3.4. Results

3.3.4.1. During hyperthermia

Comparison of mean percentage of aniline blue positive spermatozoa i.e. spermatozoa that took up the stain in the head region, with the control value (13±0.4) revealed an apparent increase as early as D34 which reached significant level at D73 (23±4) and appeared higher till the end of hyperthermia period. Due to drastic reduction in the number of spermatozoa we could not perform the technique at D95 (Fig 18).

3.3.4.2. After hyperthermia

After cessation of hyperthermia the mean percentage of aniline blue positive spermatozoa remained higher, but non-significant due to small number of volunteers, than the control at D9 (22, n=1), D20 (38.5±21.5, n=2), D34 (25±4, n=4, p=0.06), D45 (49±13, n=3), D73 (23.5±5, n=4, p=0.06) and appeared significantly higher at D95. The mean percentage value of aniline blue spermatozoa then returned to control value (before hyperthermia) when the last examination was performed at the end of follow up (Figure 18).

At D4 after cessation of hyperthermia the technique was not realised in any of the volunteers because at D120 of the hyperthermia period two volunteers were azoospermic while the rest had rare spermatozoa in their ejaculates which we preferably took for DNA fragmentation analysis (SCSA).

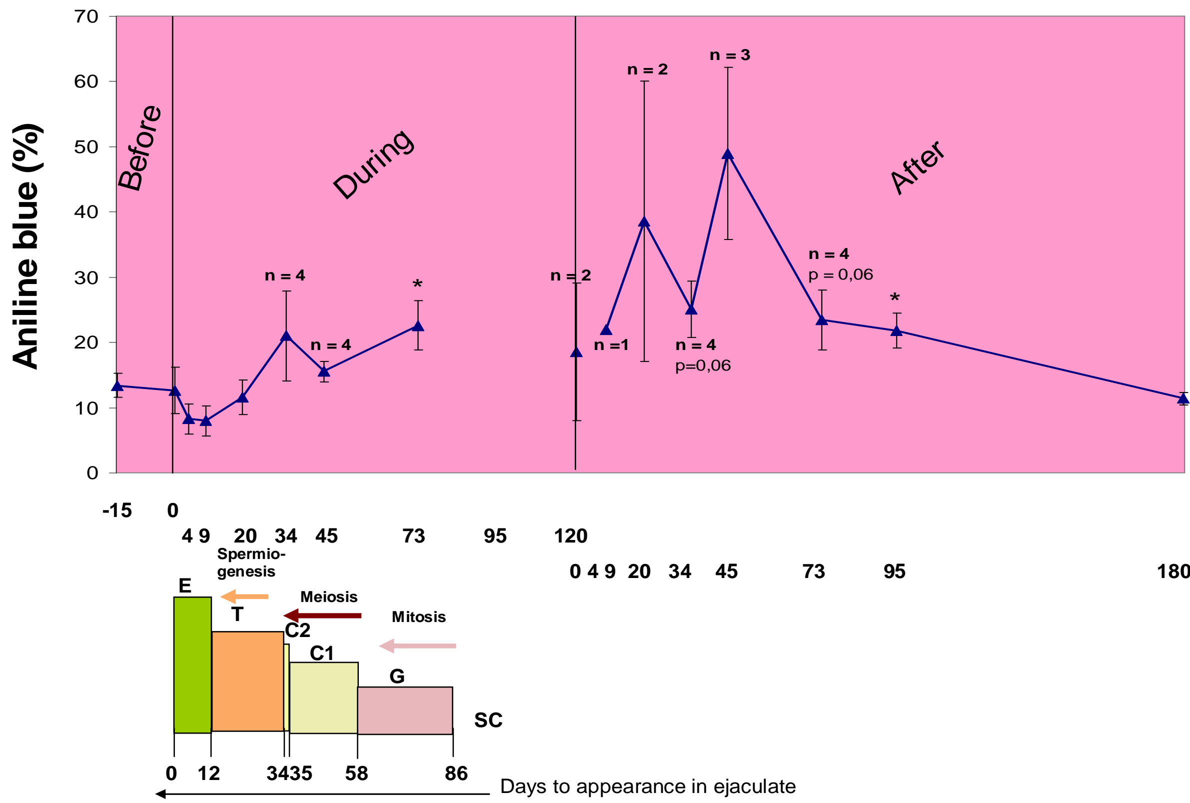

Figure 18. (A) Mean percentage of aniline blue positive spermatozoa before, during and after a mild induced testicular and epididymal hyperthermia in men. Means ± SEM (13±0.4) of the two means ± SEM before hyperthermia were compared with the means ± SEM of each data point during and after hyperthermia. However, for easier interpretation of the data both means before hyperthermia are plotted in the figure. *p<0.05, n = number of volunteers**. (B)** Represents the location and evolutionary stages of sperm during the spermatogenic process at induction of hyperthermia (D0) and their expected appearance in ejaculates. The bars represent E epididymal sperm, T spermatids, C2 spermatocytes II, C1 spermatocytes I, G spermatogonia, SC stem cells.

3.3.5. Discussion

The results of aniline blue revealed an increasing trend in the percentage of spermatozoa with immature chromatin as early as D34 during hyperthermia and this tendency persisted till D95 after cessation of hyperthermia and then returned to control value at the end of the study follow up (D180). However, the difference became significant ($p<0.05$) only when the number of volunteers assessed for aniline blue was five and it approached near significant level ($p = 0.06$) when the number of volunteers was four. Therefore, from these findings we can speculate that the testicular and epididymal hyperthermia (even small temperature rise i.e. 2 °C) impairs the maturation process of sperm chromatin. We do not know how this heat exposure modulates the maturity procedure of sperm chromatin but we know that sperm chromatin maturation takes place in round and elongated spermatids in the testes and is completed in the epididymis. It is generally believed that the main event of sperm chromatin maturation that may affect the staining properties of sperm chromatin and its maturation process depends on properly sequenced replacement of histones by transition protamines and later on by more basic protamines. The final more compact packaging of the DNA is accomplished by cross-linking of protamine disulfide bonds (Erenpreiss *et al.*, 2001). This has been reported that higher temperature causes alterations in histones to protamines ratio (Evenson *et al.,* 2000).

Dadoune *et al.,* (1988) have shown that in abnormal human spermatozoa histones may be partially replaced by protamines due to which the sperm chromatin remains loosely packed which can be shown by the aniline blue test (Dadoune *et al.*, 1988). Our results of raised percentage of aniline blue positive spermatozoa from D34 onward correspond to the fact of histones replacement during round spermatids stage (Erenpreiss *et al.*, 2001), as at D34 the ejaculated spermatozoa were most probably at the initial stage of round spermatids which have to pass the whole spermiogenesis, thus impaired by the heat stress. Similarly, the

ejaculated spermatozoa at D45 were at spermactocytes stage at the time of heat induction and they had to pass spermatocytes and spermiogenesis stages as well as epididymal transit. We did not find any augmentation in the percentage of aniline blue positive spermatozoa at D20 the time when the ejaculated spermatozoa were at elongated spermatids stage. This might be because the degree of hyperthermia induced (2 °C) was not high enough to affect the maturation process of chromatin at this stage.

In contrast to our results of DNA fragmentation, sperm count, motility, viability and morphology, which returned to their respective control values maximum by D73 after cessation of hyperthermia, aniline blue test was higher than the control till D95 after the stoppage of hyperthermia. This shows that although sperm DNA fragmentation recovers after stopping hyperthermia, the sperm chromatin maturation process (replacement of histones with protamines) still remained affected which may highlight the significance of the aniline blue test in male infertility diagnosis. A clear association between sperm chromatin and male infertility has been shown by the results of aniline blue test comparing infertile men with that of controls. When pathology of the infertile men was taken into account a significant higher percentage of stained spermatozoa was found in cases of varicoele, idiopathic infertility and patients with a previous history of unilateral cryptorchidism (Foresta *et al.*, 1992). However, controversy regarding the correlation between the percentage of aniline blue stained spermatozoa and other sperm parameters does exist as immature sperm chromatin may or may not correlate with asthenozoospermic samples and abnormal morphology patterns (Hammadeh *et al.*, 2001). The key finding is that chromatin condensation as assessed by aniline blue staining appears a good predictor for IVF outcome, although it can not determine the fertilization potential, embryonic cleavage and the rate of pregnancy obtained by intracytoplasmic sperm injection (ICSI).

In summary, we have shown the effects of a mild induced increase in testicular and epididymal temperature on sperm chromatin condensation in men. These results emphasize that aniline blue test is efficient enough to differentiate between the mature and immature sperm nuclei. This is the first report of aniline blue test on human spermatozoa after a mild induced testicular and epididymal hyperthermia, subject to further investigation, this test might be used in unexplained male infertility evaluation.

GENERAL DISCUSSION AND CONCLUSIONS

It has been a century since the opening studies were performed on increased testes temperature and its consequences on male fertility. Since then a body of evidence has been published showing the deleterious effects of hyperthermia on testes functioning and spermatogenesis impairment both in animals and men. However, the focus of earlier studies remained mainly on sperm parameters such as sperm count, motility, morphology and viability and less attention was given to the sperm chromatin. Although, reasonable data are now reported on animal models establishing the negative impact of hyperthermia on sperm chromatin integrity, early embryo development and miscarriages, yet, no study has been realised in men on this issue. In this context we report the first study in men on mild induced testicular and epididymal hyperthermia and its potential consequences on sperm chromatin integrity.

In addition to sperm chromatin integrity we also studied the effects of hyperthermia on sperm parameters in accordance with the chronology of human spermatogenesis. Our results showed that even a mild increase (≤ 2 °C) in testes and epididymal temperature adversely affects the sperm chromatin and that the effects on chromatin appear before any drop in sperm count.

Sperm DNA fragmentation occurred as early as D20 of hyperthermia and persisted as long as hyperthermia persisted. It started to return to control values from D45 after the cessation of hyperthermia and reached control value at D73 after hyperthermia. Similar pattern of alterations was observed for HDS except that it differed from the control as early as D34 of hyperthermia. Further assessment of sperm chromatin by aniline blue test revealed a non-significant change compared with control as early as D34 of hyperthermia which remained elevated throughout the entire period of hyperthermia and returned to control values later (D180) than sperm DFI and HDS (D73).

The results of SCSA emphasize that spermatocytes and spermatids were the most affected cells by hyperthermia which is in agreement with the previous studies on mice (Da Vita *et al.,* 1990; Sailer *et al.,* 1997; Setchell, 1998; Paul *et al.,* 2008b; Perez-Crespo *et al.,* 2008), rats (Chowdhury and Steinberger, 1964; Blackshaw and Hamilton, 1971; Blackshaw *et al.*, 1973), rabbits (Kong *et al.,* 2000) and monkeys (Zhang *et al.,* 2003). The DNA damage was more profound at D34 and D45 compared with the damage at D20 during hyperthermia. This marked increase in sperm DFI and HDS at D34 and D45 was most probably because at these stages (spermatocytes or early spermatids) DNA strands are not firmly bound and are less dense than in late spermatids (D20) which is in accordance with the study of Paul *et al.,* (2008b) in mice where most affected cells were the spermatocytes and spermatids after scrotal heating. The increase in sperm DNA fragmentation could further be elaborated in relation to the chronology of spermatogenesis such as: (a) at D20, hyperthermia affected the last phase of spermiogenesis (elongated spermatids), when nuclear compaction takes place and the majority of nuclear proteins (histones) are replaced with protamines; (b) at D34, heat affected the cells that were at late spermatocytes or early spermatids stages. They travelled the whole spermiogenesis and epididymal transit period thus were exposed to the heat longer than the cells at D20; (c) at D45, the cells were at primary spermatocytes stages and travelled the rest of spermatocytes stages, whole spermiogenesis and epididymal transit and, were exposed to heat stress longer than both D20 and D34. In conclusion, the longer the time of mild heat stress the more damaging effects on the spermatozoa.

It is noteworthy that we observed alterations in sperm chromatin integrity when the sperm count was still compatible with natural conception. Damaged sperm DNA may have a negative impact on the fate of the embryo both in natural and assisted reproduction. Some authors have reported reduced blastulation rates after in vitro fertilization (Seli *et al.,* 2004)

and increased frequency of miscarriages (Virro *et al.,* 2004; Borini *et al.,* 2006; *Zini et al.,* 2008) in women whose partners had high sperm DNA damage compared with women whose had low damage. Though, in the above studies hyperthermia was not the cause of DNA damage, nevertheless, the effects of hyperthermia on sperm chromatin integrity and fate of embryo are well established in animal studies. Therefore, the present study, being the first in men, provides a solid evidence that mild induced hyperthermia does alter the sperm chromatin integrity which may have negative impact on the future of the embryo.

In accordance with the previous studies we demonstrated that hyperthermia caused reduction in sperm count, motility, viability and morphology. As in the previous studies the results were not interpreted according to the chronology of human spermatogenesis our findings give more explanation on stage specific effects of hyperthermia and the possible arrest of spermatogenesis cycle during hyperthermia. For example, sperm collected at D4 and D9 were present in the epididymis when hyperthermia was induced and no change in individual sperm parameters was observed at these points as demonstrated previously (Mieusset *et al.,* 1992a; Perez-Crespo *et al.,* 2008). However, irrespective of the fact that individual sperm morphology defects were not different than controls at D9 of hyperthermia, multiple anomalies index (MAI) was significnalty higher at this stage. Sperm motility fell down significantly at D20 compared with control while drop in sperm count and percentage of viable spermatozoa differed significantly at D34 during hyperthermia. From D45 during hyperthermia till D34 after hyperthermia these parameters were significantly lower than the controls and may represent the male contraception zone where all volunteers were severe oligospermic. Additionally, when thinking of male contraception, the inhibition and recovery phases of spermatogensies must be carefully addressed as DNA damage occurred earlier than any drop in sperm count (D20).

After cessation of hyperthermia total sperm count reached similar to control value at D73 which suggests a possible arrest of spermatogenic cycle at spermatogonia B or early spermatocytes stage. Because, at D73 after hyperthermia the spermatogenic cycle was not yet completed (73 - 12 epididymal transit time = 61 days). This is in accordance with the findings observed in rabbits after induced unilateral cryptorchidism for one spermatogenic cycle, showing no significant influence of abdominal temperature on spermatogonia compared with the control testis but the primary spermatocytes were arrested at the G2 to M phase transition (Kong *et al.*, 2000). Similarly, earlier histological studies also showed the effect of heat more specifically on primary spermatocytes (Setchell, 1998).

We speculate that at spermatocyte stage some cells underwent apoptosis some appeared as round cells; a few continued to develop into spermatozoa while others became arrested in a "frozen state". As spermatogonia continued dividing and differentiating, several waves of germ cells accumulated as late spermatogonia B and spermatocytes in the "frozen state"; finally, when heating was stopped, all arrested germ cells restarted their evolutionary process together giving a sperm output similar to control value at D73 after hyperthermia. This concept is further supported by the studies in rodents where scrotal hyperthermia (43°C for 15 minutes) (McLean *et al.,* 2002) and vitamin A withdrawal (van Pelt and de Rooij, 1990) resulted in spermatogenesis arrest at spermatogonia stage in mice, whereas at preleptotene spermatocytes in rats (Huang and Hembree, 1979).

In short, spermatogenesis is impaired when a condition of moderate heat stress occurs and when the heat stress is withdrawn the spermatogenesis resumes. This implicates that during male fertility explorations such conditions of stress may not be neglected and should be treated before going for assisted reproduction.

Conclusions and prospectives

Our model of diurnal artificial crypotorchdism provides a feasible, reversible and an experimental approach to investiagte the alterations in sperm chromatin. The results obtained demonstrate that even a mild increase in testes and epididymal temperature does impair the sperm chromatin integrity before any drop in sperm count. Therefore, potential interest involves male contraception specifically during the inhibition and recovery phases of spermatogenesis under such mehthod. Moreover, to date, these findings may have clinical implications in male infertility, repeated miscarriages without female factor involved as well as assisetd reproductive technologies.

In prospective, the samples collected and freezed during this study will help to analyse the sperm aneuploidy, proteomics of sperm and seminal plasma. Such investigations will improve the understanding and reasoning of the alterations in spermatogenesis, epididymal functioning and male gamete parameters. Additionally this could lead to the indentification of molecules that can be targets for therapeutic and diagnostic tools in male infertility and contraception.

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ANNEXES

EQUIPE ACCUEIL / RESEARCH TEAM N° 3694

Recherche en Fertilité Humaine

Human Fertility Research Group

Effet d'une augmentation de la température testiculo-épididymaire sur la fragmentation de l'ADN du spermatozoïde chez l'homme

TEMPTESTIS

CAHIER D'OBSERVATION

Initiales du patient : |____|____| |____|____|

(les deux 1er lettres du nom et les deux 1er lettres du prénom)

Numéro d'enregistrement dans l'étude : /_0__/__9_/_0__/_6_/

(Numéro d'enregistrement :code CECOS+numéro d'ordre dans l'étude)

EA 3694 FERTILITE HUMAINE

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C 1ère consultation : pré-inclusion

Date : ……. / ……. / …….

Questionnaire Antécédents rempli lors de la consultation	
ATCD Fertilité (famille et hors couple actuel**)**	
Nombre de frères	…………………..
Nombre de sœurs	…………………..
Stérilité famille	☐ **non** ☐ frère ☐ père ☐ oncle paternel ☐ oncle maternel ☐ autre paternel ☐ autre maternel
Si besoin préciser :	
Cryptorchidie famille	☐ **non** ☐ frère ☐ père ☐ oncle paternel ☐ oncle maternel ☐ autre paternel ☐ autre maternel
Si besoin préciser :	
FCS mère avant la naissance de Monsieur	☐ ne sait pas ☐ **non** ☐ doute ☐ oui
Prise de médicaments lors de grossesse mère	☐ ne sait pas ☐ **non** ☐ doute ☐ oui
Fécondité Monsieur antérieure au couple actuel	☐ **non** ☐ FCS ☐ GEU ☐ IVG ☐ IMG ☐ ENFANTS ☐ non réponse
Age dernière grossesse hors couple actuel (quel qu'en ait été l'issue)	…………………..
Patient célibataire	☐ oui ☐ **non** ☐ non réponse (si oui, sauter 10 questions)
ATCD Fertilité couple actuel	
Durée (années) de vie commune	……………ans..

Couple nombre d'enfants	……………………
Couple âge du dernier enfant	……………………
Grossesse non menée à terme dans le couple	☐ **non** ☐ FCS ☐ GEU ☐ IVG ☐ ITG ☐ non réponse
Durée (mois) avant 1ère conception (délai nécessaire pour concevoir)	……………mois..
Durée (mois) depuis dernière conception	……………mois..
Avez-vous essayé d'avoir un enfant sans succès	☐ **non** ☐ oui si non sauter 3 questions
Avez-vous déjà consulter pour infécondité	☐ **non** ☐ oui
Avez-vous déjà réalisé un spermogramme	☐ **non** ☐ oui
Durée (mois) infécondité dans le couple	……………mois..
Erection	☐ normale ☐ anormale
Ejaculation	☐ normale ☐ anormale ☐ anéjaculation
ATCD infectieux : pour l'uréthrite : avez vous eu un écoulement, des brûlures et avez vous consulté et été traité pour cette affection, pour l'épididymite ou orchi-épididymite : avez vous été consulté ou traité pour une douleur importante avec fièvre …. En général ceux qui en ont eu le savent, des précisions que vous jugerez utiles peuvent être rajoutées à la fin du questionnaire	
ATCD oreillons	☐ ne sait pas ☐ **non** ☐ durant l'enfance ☐ adulte sans orchite ☐ adulte avec orchite unilatérale ☐ adulte avec orchite bilatérale
ATCD infection urinaire	☐ **non** ☐ oui
ATCD infection urinaire année dernier épisode	……………………
ATCD Uréthrite/ blenno	☐ **non** ☐ oui

ATCD uréthrite/blenno année dernier épisode	……………………
ATCD épididymite Ou orchiépididymite	☐ **non** ☐ droit ☐ gauche ☐ les deux
ATCD épididymite ou orchiépididymite année dernier épisode	……………………
ATCD Chirurgicaux	
ATCD cryptorchidie	☐ oui ☐ **non** si non sauter 8 questions
Cryptorchidie droite	☐ **non** ☐ traitée médicalement ☐ traitée chirurgie ☐ descendu spontanément ☐ ne sait pas
Cryptorchidie gauche	☐ **non** ☐ traitée médicalement ☐ traitée chirurgie ☐ descendu spontanément ☐ ne sait pas
Age lors de la descente spontanée DROITE	……………………
Age lors de la descente spontanée GAUCHE	……………………
Age lors du traitement médical DROIT	……………………
Age lors du traitement médical GAUCHE	……………………
Age lors de la chirurgie crypto DROITE	……………………
Age lors de la chirurgie crypto GAUCHE	……………………
ATCD chirurgical	☐ **non** ☐ vessie ☐ bassin-périné trauma ☐ appendicectomie ☐ rectum ☐ prostate ☐ sonde à demeure ☐ hypospadias ☐ autre
ATCD trauma. scrotal	☐ **non** ☐ droit ☐ gauche ☐ bilatéral

(oedème - hématome)	
Année du traumatisme	…………………………
ATCD chirurgical DROIT	☐ **non** ☐ épididyme ☐ déférent ☐ torsion du cordon ☐ biopsie testis ☐ orchidectomie cancer ☐ orchidectomie crypto ☐ orchidectomie autre ☐ Hydrocèle vaginale ☐ autre
ATCD chirurgical GAUCHE	☐ **non** ☐ épididyme ☐ déférent ☐ torsion du cordon ☐ biopsie testis ☐ orchidectomie cancer ☐ orchidectomie crypto ☐ orchidectomie autre ☐ Hydrocèle vaginale ☐ autre
Date de l'orchidectomie pour cancer testis :	Jour : -- mois : -- année :----
ATCD hernie inguinale	☐ **non** ☐ droite ☐ gauche ☐ bilatérale si non sauter 2 questions
Age chirurgie hernie inguinale DROITE	…………………..
Age chirurgie hernie inguinale GAUCHE	…………………..
ATCD varicocélectomie	☐ **non** ☐ droit ☐ gauche ☐ bilatérale
Cure varicocèle	☐ chirurgicale ☐ embolisation ☐ les deux
Année varicocélectomie	…………………..
ATCD médicaux et exposition	
ATCD médical	☐ **non** ☐ diabète ☐ sinusites chroniques ☐ pathologies respiratoires chroniques ☐ mucoviscidose ☐ neurologique ☐ insuffisance rénale ☐ Tuberculose ☐ autre
Prise de médicaments dans l'année écoulée	☐ **non** ☐ **oui**
Prise de médicament dans l'année écoulée	☐ antibiotiques ☐ corticoïdes ☐ androgènes ☐ acide rétinoïque ☐ hormones hypophysaires ☐ neuroleptiques ☐ anxyolytiques antidépresseur ☐ anticomitiaux ☐ autres

Prises médicaments dans les trois derniers mois (inscrire nom du médicament et date de la dernière prise)	Nom :------------------------------------- Date :-------------------------------------- Nom :--------------------------------------- Date :-- Nom :--- Date :-- Nom :-- Date :--
Dernière fièvre > 38°C	☐ moins de 3 mois ☐ plus de 3 mois ☐ non réponse
Prenez vous des bains très chaud (>40°C)	☐ non ☐ oui combien de fois par semaine :
Consommation d'alcool	☐ jamais ☐ quelquefois ☐ < 1 litre/jour ☐ > 1 litre/jour
Nbre cigarettes par jour (ou équivalent)	……………………
Tabagisme passif (à la maison ou au travail):	☐ non ☐ oui
Drogues récréatives :	☐ non ☐ oui
Drogues :	☐ Cannabis ☐ Ectasy ☐ LSD ☐ cocaine ☐ héroine ☐ colle ☐ autre
Drogues	☐ occasionnel (moins d'une fois le mois) ☐ régulier
Drogues si régulier :	Tous les x jours : ----
Lieu habitation :	☐ grande ville (+100 000 habitants) ☐ petite ville (<) ☐ zone rurale
Profession	*Texte libre*
Nombre d'heures en position assise par jour	……………………
Nbre heures/jour exposition > 37°C profession	……………………
Exposition intermittente à T° ambiante >	☐ oui ☐ **non** ☐ non réponse

37°C	
ATCD d'exposition à	☐ **non** ☐ radiations ☐ bruit (> 100db) ☐ toxiques ☐ pesticides insecticides ☐ autres ☐ non réponse
Autres précisions Apporter toute précision que vous jugerez utile notamment sur l'exposition	*texte libre*
Conclusion Existence d'une exposition ou d'un facteur de risque (ATCD)	☐ **non** ☐ oui ☐ doute

	EXAMEN CLINIQUE		
Date consultation	........../.........../ 200..		
Médecin examinateur	☐ Dr R. Mieusset ☐ Dr L. Bujan		
Gynécomastie	☐ oui	☐ **non**	☐ non réponse
Caractères sexuels normaux	☐ oui	☐ **non**	☐ non réponse
Androgénisation	☐ normale ☐ autre	☐ aspect gynoïde	☐ pilosité anormale ☐ non réponse
Scrotum	☐ court	☐ moyen	☐ long ☐ non réponse
Epaisseur scrotum	☐ fin	☐ épaissi	☐ non réponse
Testicules	☐ les 2 présents ☐ les 2 absents	☐ absence gauche	☐ absence droit ☐ non réponse
Panicule adipeux	☐ **non** ☐	☐ oui +	☐ oui ++ ☐ non réponse
Remontée possible dans canal inguinal	☐ **non** ☐ les deux	☐ à droite	☐ à gauche ☐ non réponse
Ressentez-vous vos testis en position haute droite	☐ jamais ☐ souvent	☐ rarement	☐ parfois ☐ non réponse
Ressentez-vous vos	☐ jamais	☐ rarement	☐ parfois

testis en position haute gauche	☐ souvent ☐ non réponse
Position testiculaire droit	☐ bas ☐ haut ☐ intracanalaire ☐ absent ☐ non réponse
Position testiculaire gauche	☐ bas ☐ haut ☐ intracanalaire ☐ absent ☐ non réponse
Testis droit longueur	(de 0,0 à 60,0)
Testis droit largeur	(de 0,0 à 60,0)
Volume testis droit	(ml)
Testis gauche longueur	(de 0,0 à 60,0)
Testis gauche largeur	(de 0,0 à 60,0)
Volume testis gauche	(ml)
Température antérotesticulaire droite	(C°) (de 32,0 à 36,8)
Température antérotesticulaire gauche	(C°) (de 32,1 à 36,8)
Température intertesticulaire	(C°) (de 32,1 à 36,8)
Température rectale	(C°) (de 36,0 à 41,0)
Température salle d'examen	(C°) (de 17,0 à 30,0)
Différentiel recto-scrotal droit	(C°)
Différentiel recto-scrotal gauche	(C°)
Hydrocèle	☐ **non** ☐ droit ☐ gauche ☐ bilatéral ☐ non réponse
Kystes du cordon	☐ **non** ☐ droit ☐ gauche

	☐ bilatéral ☐ non réponse
Varicocèle droit	☐ **non** ☐ vasalva + ☐ petit ☐ gros ☐ non réponse
Type varicocèle droit	☐ cordonal ☐ scrotal ☐ les deux ☐ 1 ☐ non réponse
Varicocèle gauche	☐ **non** ☐ vasalva + ☐ petit ☐ gros ☐ non réponse
Type varicocèle gauche	☐ cordonal ☐ scrotal ☐ les deux ☐ 1 ☐ non réponse
Déférent droit	☐ normal ☐ nodulaire ☐ induré ☐ épaissi ☐ absent total ☐ absent partiel ☐ doute ☐ non réponse
Déférent gauche	☐ normal ☐ nodulaire ☐ induré ☐ épaissi ☐ absent total ☐ absent partiel ☐ doute ☐ non réponse
Position épididyme droit	☐ normal ☐ inversé en hauteur ☐ inversé latéral. ☐ éloigné ☐ absent queue ☐ absent corps ☐ absent tête ☐ non réponse
Position épididyme gauche	☐ normal ☐ inversé en hauteur ☐ inversé latéral. ☐ éloigné ☐ absent queue ☐ absent corps ☐ absent tête ☐ non réponse
Palpation épididyme droit	☐ normal ☐ kystique ☐ nodulaire ☐ épaissi ☐ induré ☐ turgescent ☐ non réponse
Localisation des anomalies épididyme D	☐ tête ☐ corps ☐ queue ☐ non réponse
Palpation épididyme gauche	☐ normal ☐ kystique ☐ nodulaire ☐ épaissi ☐ induré ☐ turgescent ☐ non réponse
Localisation des anomalies épididyme G	☐ tête ☐ corps ☐ queue ☐ non réponse
Conclusion examen clinique	

andro.	
Conclusion examen clinique	☐ normal ☐ anormal

Données validées par (nom prénom) : ……………………………………

Date de la validation : /__/__/ /__/__/ /__/__/__/__/

CJ0

Date : ……. / ……. / …….

Le médecin expliquera le port du sous vêtement

.

Un examen andrologique doit être réalisé s'il n'a pas été réalisé antérieurement (noter les données dans la première grille cf C1 pré-inclusion)

Ce jour est le début de l'induction de l'hyperthermie

Problèmes particuliers :

Attitude corrective apportée :

Observation Remarques :

Spermogramme J0

Date *(jour/mois/année)* : /__/__/ /__/__/ /__/__/__/__/

Délai d'abstinence *(jours)* : /__/__/__/

Volume *(ml)* : /__/__/, /__/

pH : /__/, /__/

Viscosité *(0= Normale , 1=Anormale)* : /___/

Concentration des spermatozoïdes *(10^6/ml)* : /__/__/__/__/__/ , /__/

Concentration des cellules rondes *(10^6/ml)* : /__/__/ , /__/

Polynucléaires *le cas échéant (10^6/ml)* : /__/__/ , /__/

Pourcentage de spermatozoïdes mobiles à 1 heure:

Mobilité a : /__/__/

Mobilité b : /__/__/

Mobilité c : /__/__/

Mobilité d : /__/__/

Vitalité *(%de vivants)* : /__/__/

Lame spermocytogramme

Numéro de congélation (code de la paillette) : /__/__/__/__/__/__/__/__/__/__/__/

(Lettre/N° CECOS/Année/N° rang congélation)

Couleur paillette : /____________________/

Nombre de paillettes congelées : /__/__/

Nombre de spermatozoïdes par paillette *(10^6/ml)* : /__/__/ , /__/

Nombre tube plasma séminal pour l'étude : /__/__/

Aliquote Fixés ☐ non ☐ oui nombre /__/__/

Protéomique ☐ oui

Données validées par (nom prénom) : ……………………………………

Date de la validation : /__/__/ /__/__/ /__/__/__/__/

C J0

Date : ……. / ……. / …….

Le médecin expliquera de nouveau le port du sous vêtement

.

Un examen andrologique doit être réalisé s'il n'a pas été réalisé antérieurement (noter les données dans la première grille cf C1 pré-inclusion)

Ce jour est le début de l'induction de l'hyperthermie

Problèmes particuliers :

Attitude corrective apportée :

Observations remarques :

C J120 (suite)

Date : ……. / ……. / …….

.

Observance :

Problèmes particuliers :

Attitude corrective apportée :

Observations remarques :

Spermogramme J120

Date *(jour/mois/année)* : /__/__/ /__/__/ /__/__/__/__/

Délai d'abstinence *(jours)* : /__/__/__/

Volume *(ml)* : /__/__/, /__/

pH : /__/, /__/

Viscosité *(0= Normale , 1=Anormale)* : /___/

Concentration des spermatozoïdes *(10^6/ml)* : /__/__/__/__/ , /__/

Concentration des cellules rondes *(10^6/ml)* : /__/__/ , /__/

Polynucléaires *le cas échéant (10^6/ml)* : /__/__/ , /__/

Pourcentage de spermatozoïdes mobiles à 1 heure:

Mobilité a : /__/__/

Mobilité b : /__/__/

Mobilité c : /__/__/

Mobilité d : /__/__/

Vitalité *(%de vivants)* : /__/__/

Lame spermocytogramme

Numéro de congélation (code de la paillette) : /__/__/__/__/__/__/__/__/__/__/

(Lettre/N° CECOS/Année/N° rang congélation)

Couleur paillette : /____________________/

Nombre de paillettes congelées : /__/__/

Nombre de spermatozoïdes par paillette *(10^6/ml)* : /__/__/ , /__/

Nombre tube plasma séminal pour l'étude : /__/__/

Aliquote Fixés ☐ non ☐ oui nombre /__/__/

Protéomique ☐ oui

Données validées par (nom prénom) : ……………………………………

Date de la validation : /__/__/ /__/__/ /__/__/__/__/

C J300 (J180) (suite)

Date : ……. / ……. / …….

.

Problèmes particuliers :

Attitude corrective apportée :

Observations remarques :

Spermogramme J300 (J180)

Date *(jour/mois/année)* : /__/__/ /__/__/ /__/__/__/__/

Délai d'abstinence *(jours)* : /__/__/__/

Volume *(ml)* : /__/__/, /__/

pH : /__/, /__/

Viscosité *(0= Normale , 1=Anormale)* : /___/

Concentration des spermatozoïdes *(10^6/ml)* : /__/__/__/__/__/ , /__/

Concentration des cellules rondes *(10^6/ml)* : /__/__/ , /__/

Polynucléaires *le cas échéant (10^6/ml)* : /__/__/ , /__/

Pourcentage de spermatozoïdes mobiles à 1 heure:

Mobilité a : /__/__/

Mobilité b : /__/__/

Mobilité c : /__/__/

Mobilité d : /__/__/

Vitalité *(%de vivants)* : /__/__/

Lame spermocytogramme

Numéro de congélation (code de la paillette) : /__/__/__/__/__/__/__/__/__/__/__/

(Lettre/N° CECOS/Année/N° rang congélation)

Couleur paillette : /____________________/

Nombre de paillettes congelées : /__/__/

Nombre de spermatozoïdes par paillette *(10^6/ml)* : /__/__/ , /__/

Nombre tube plasma séminal pour l'étude : /__/__/

Aliquote Fixés ☐ non ☐ oui nombre /__/__/

Protéomique ☐ oui

Données validées par (nom prénom) : ……………………………………

Date de la validation : /__/__/ /__/__/ /__/__/__/__/

Observation Remarques :

Ne pas oublier de mettre la date avant d'écrire :

Formulaire de consentement

Etude : TEMPTESTIS
Effet d'une augmentation de la température testiculo-épididymaire sur la fragmentation de l'ADN du spermatozoïde chez l'homme

Promoteur : CHU Toulouse
Investigateur-Coordonnateur : Dr Louis Bujan, PH-MCU (05 67 77 14 35)
Co-Investigateur principal : Dr Roger Mieusset, PH-MCU

Je soussigné
Nom : ______________________
Prénom : ____________________
Date et lieu de naissance : ____________________________________
Adresse : ____
N° de sécurité sociale : _________________________________

Accepte de participer au protocole de recherche référencé ci dessus.

J'ai bien compris l'objet du protocole qui est de créer une augmentation de la température testiculo-épididymaire grâce au port d'un sous vêtement adapté durant les heures d'éveil et ce durant 4 mois. Des consultations avec les médecins investigateurs de l'étude auront lieu avant, pendant et après la réalisation de l'augmentation de la température. Ces consultations me permettront d'apprendre la méthode d'augmentation de la température et permettront un suivi au cours du protocole. J'ai bien compris que pour l'étude des effets de cette augmentation de la température je devrais faire des recueils de sperme , précédés d'une période d'abstinence de 3 à 6 jours, avant le port du sous vêtement adapté (3 recueils), pendant (8 recueils) et après la période de port (8 recueils). Ces recueils de sperme sont indispensable dans le cadre de l'étude et seront réalisés par masturbation au laboratoire.

J'accepte que les échantillons de sperme obtenus dans les conditions prévues au protocole puissent être soumis aux analyses prévues dans l'étude et que les prélèvements puissent être congelés, stockés dans le centre de ressources biologiques GERMETHEQUE en vue d'analyses ultérieures dans le cadre de la recherche des effets de l'augmentation de la température testiculaire sur les caractéristiques du sperme et des spermatozoïdes ou de recherche en fertilité humaine. Ces prélèvements seront conservés jusqu'à épuisement au sein du CRB suivant la notice d'information du CRB dont j'ai pris connaissance.

J'ai bien compris que ma participation au protocole n'entraînait pas d'actes médicaux invasifs (ponctions…) mais seulement la réalisation de recueils de sperme. L'indemnité de 3500€ prévue dans le cadre de l'étude me sera versée suivant l'échéancier suivant : 500€ à J0, 500€ à J34, 300€ à J120, 300€ à J 154, 1900€ à J300. Les frais liés à la recherche sont pris en charge par le promoteur.

J'ai par ailleurs été informé que j'étais libre de me retirer du protocole à tout moment et je donne mon accord pour que les données me concernant soient stockées, de manière anonyme. Concernant les données nominatives médicales détenues dans mon dossier

médical je suis informé que je bénéficie d'un droit d'accès à tout moment, soit directement, soit par l'intermédiaire du médecin de mon choix.

J'atteste ne pas être sous un régime de protection des majeurs et être affilié à un régime de sécurité sociale.

Je déclare avoir été informé oralement du protocole de recherche et avoir pris connaissance de la lettre d'information qui m'a été remise.

Signature du patient : ___________________________

Je consens à ce qu'une étude sur le spermatozoïde, y compris génétique, à l'exclusion de toute fécondation soit réalisée sur mes prélèvements, conformément à la thématique de la recherche.

Signature du patient : ___________________________

Je consens à ce qu'une partie du prélèvement soit destinée au centre de ressources biologique GERMETHEQUE en vue de constituer une collection pour des études ultérieures dans le champ de la fertilité humaine.
Cette collection sera conservée pour faire des recherches scientifiques liées aux altérations du spermatozoïde, incluant des recherches sur les facteurs génétiques. GERMETHEQUE a été déclarée auprès des instances conformément à la réglementation en vigueur.
Si une nouvelle étude devait être envisagée, dont l'objectif ne serait pas dans le champ de la fertilité humaine, objectif de la collection, celle-ci devrait faire l'objet d'un nouveau protocole de recherche soumis pour avis au CPP, dont vous seriez informé et pour lequel nous vous demanderions un nouveau consentement.

Signature du patient : ___________________________

Fait à ……………………………………., le ………. / ………. / ……….

Nom et signature du médecin : _____________________________

Trois exemplaires dont un pour le patient, un pour l'équipe médicale et un pour le CRB
Ce consentement doit être conservé dans la plus stricte confidentialité et sécurité avec les consentements pour le don