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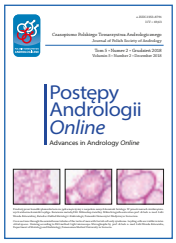

HYPERTHERMIA AND SPERM QUALITY – A RISK FACTOR FOR MALE INFERTILITY OR CONTRACEPTIVE TARGET

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Abstract

Due to growing male infertility, many questions have arisen in recent years about the cause and mechanism of its formation. It is commonly accepted that body temperature can be harmful to male fertility. It is particularly visible in men with varicocele and history of cryptorchidism in childhood. Numerous studies regarding testicular hyperthermia have been carried out in animal models, but the underlying mechanism for reduced fertilizing potential as a consequence of genital heat remains not fully explained. The main reason for this situation is a relatively small number of controlled prospective studies with regard to various heat stress as well as a wide range of analyzed seminal parameters. Recently published data suggest the participation of the four possible mechanisms involved in heat-induced germ cell damage, including oxidative stress response, sperm death, epigenetic modifications and immune/autoimmune reactions. This review is an attempt to summarize current knowledge of the main pathophysiological concepts constituting a link between internal as well as external genital heat stress and male fertility/infertility status.

Key words: hyperthermia, sperm quality, oxidative stress, apoptosis, contraception

Abbreviations

AsA – antisperm antibodies; Bax – proapoptotic protein; Bcl2 – B-cell leukemia/lymphoma 2; Cu,ZnSOD – copper, zinc superoxide dismutase; DIABLO - mitochondria-derived activator of caspases; FAS – Fas cell surface death receptor; HNRNPH1 – heterogeneous nuclear ribonucleoprotein H1; *HSPA1B* – heat shock protein family A (Hsp70) member 1B gene; IAPs – inhibitor of apoptosis proteins; IL-1α – interleukin 1α; IL-1β – interleukin 1β; IL-6 – interleukin 6; IL-8 – interleukin 8; IL-10 – interleukin 10; IL-13 – interleukin 13; IL-14 – interleukin 14; IL-17A – interleukin 17A; IL-18 – interleukin 18; IL-37 – interleukin 37; MAPKs – mitogen-activated protein kinases; MAPK1/3 – mitogen-activated protein kinase 1/3; MAPK 14 – mitogen-activated protein kinase 14; MDA – malondialdehyde; miRNAs – microRNAs; miR-15a – microRNA-15a; ROS – reactive oxygen species; TGF-β – transforming growth factor β; TNF-α – tumor necrosis factor α; TRAIL – tumor necrosis factor-related apoptosis-inducing ligand

Spermatogenesis and spermiogenesis are temperature-dependent and occur properly at a minimum 2°C below the intra-abdominal temperature. The temperature within the testes is affected by the environment of the surrounding scrotal sac. According to *Skandhan* and *Rajahariprasad* (2007), scrotum maintains a lower temperature in male gonads, as a significant amount of energy is released during the spermatogenesis, which is a by-product of this process. In men under normal healthy environmental and physiological conditions, the testicular thermoregulation is able to maintain 'normal' scrotal hypothermia. Prolonged testicular exposure to elevated temperature and impaired arteriovenous testicular system could lead to chronic thermo-dysregulation which may, in time, result in reducing the quality of semen due to partial or complete blockage of spermatogenesis. In this context, the male genital heat exposure is considered to be a risk factor for male infertility (*Mieusset* and *Bujan*, 1995). Two main groups of factors potentially associated with an increased testicular temperature can be distinguished: pathophysiological factors (e.g. varicocele, cryptorchidism, obesity, fever) and environmental ones (e.g. tight clothing and sitting or sleeping postures, hot bath and sauna, cycling, sedentary working mode, working in high temperature conditions) (*Durairajanayagam et al.*, 2014). Most results of experimental and clinical studies indicate a decrease in semen quality of men exposed to the temperature factor, which may be associated with a decrease in sperm fertilizing potential; and high temperature effects are primarily associated with the damage to sperm DNA.

Effect of pathophysiological conditions associated with raised scrotal temperature on conventional sperm parameters

Varicocele and cryptorchidism may be the cause of male infertility, although the mechanism of male gametes impaired function in the course of these pathologies is multifactorial and remains largely unknown. The situation is complicated by the fact that infertile men with these diseases do not always show abnormal sperm count, motility and spermatozoa morphology. An elevated testis temperature may be a common element of fertility disorders in men with varicocele and cryptorchidism.

It is estimated that varicocele occurs in about 15% of men (mainly young ones), and this percentage increases in the group of people with fertility problems and constitutes 30–40% (*Nieschlag et al.*, 2010). A varicocele is an abnormal dilatation of venous vessels of the pampiniform plexus, which drains the testicles. Blood retention in veins as a result of the backflow causes an increase in temperature inside male gonads (*Lerchl et al.*, 1993). Many researchers hypothesized that a combination of several factors, such as hyperthermia, hypoperfusion and hypoxia, hormonal imbalance, oxidative stress, increased apoptosis and exogenous toxicants can affect spermatogenesis and sperm function in men with this pathology (*Sheehan et al.*, 2014). Males with varicocele show infertility with variable spermiogram. Moreover, some individuals with varicocele remain fertile but their fertility potential might decline gradually (*Cho et al.*, 2016). A meta-analysis of standard semen outcomes carried out by *Nork et al.* (2014) showed a significant decrease in sperm density and motility in young men with this pathology which appears to lead to improvement after surgical intervention. Some authors have also proved the association between varicocele, and decreased testicular volume, and impaired sperm quality (*Guzel et al.*, 2015; *Kurtz et al.*, 2015). However, the improvement in testis volume after varicoselectomy was not always associated with the improvement in conventional semen parameters as sperm concentration, total motile sperm count and normal morphology (*Zhou et al.*, 2015). Recently, semen quality in varicocele patients has often been correlated with the degree of sperm DNA fragmentation (*Durairajanayagam et al.*, 2015; *Jung* and *Schuppe*, 2007; *Ku et al.*, 2005). Therefore, it is postulated that surgical treatment of varicocele is associated with improvement in sperm nuclear DNA integrity (*Agarwal et al.*, 2017). The question of whether abnormal spermatozoa are an indicator of increased DNA fragmentation in this group of patients is still open. The potential mechanisms connected with the induction of sperm DNA damage which can be activated in spermatozoa of infertile men with varicocele will be discussed in detail later in this review.

Cryptorchidism (undescended testis/es) is one of the most common malformations in boys and is defined as

the abnormal location (outside the scrotum) of one or both testicles. Cryptorchidism is diagnosed in about 4% of boys born on time and in about 8% of prematurely born male children. An increase in the incidence of this abnormality has been noted in recent years. The etiology of cryptorchidism is mostly idiopathic and involves multiple genetic, hormonal and environmental factors (*Barthold et al.*, 2016). The complete descent of testes through the inguinal canal into the scrotum before birth or in the first months of life is important for the proper course of spermatogenesis and in case of its failure may lead to infertility. Complications of untreated cryptorchidism may also include testicular cancer or syndrome of gonadal dysgenesis (*Toppari et al.*, 2014). Male gonads, which remain in the abdominal cavity for a long time, are exposed to body temperature, which may affect changes in the process of spermatogenesis and the level of sex hormones (*Lee* and *Coughlin*, 2002). The degree of damage to testicular function depends on whether the cryptorchidism involves one or both testes, their position in relation to the inguinal canal and the length of time before fixation of testis/es in the scrotum (*Agoulnik et al.*, 2012). The higher incidence of azoospermia has been revealed, especially in bilateral cryptorchidism and delayed surgical intervention by orchidopexy (*Moretti et al.*, 2007). Few data available on semen quality in adult men with history of cryptorchidism are related to poor sperm concentration and motility in this group of patients (*Moretti et al.*, 2007; *Trsinar* and *Muravec*, 2009). Additionally, morphological ultrastructural studies showed statistically significant higher percentage of spermatozoa with abnormal morphology in semen of cryptorchid men compared to the group of fertile ones (*Moretti et al.*, 2007). In turn, *Hadziselimovic* (2008) observed a significant improvement in seminal parameters, including sperm morphology, in men with cryptorchidism who received combined surgical and hormonal treatment in childhood, compared to men who underwent only surgical treatment. Most probably, abnormal semen quality observed in cryptorchid men can be the result of a strong influence of the temperature factor in this pathology.

Obesity has recently become an extremely important health problem on a global scale. It was shown that men who have fertility problems are more likely to be overweight compared to fertile men (*Hammoud et al.*, 2008). The body mass index (BMI) ≥25 was associated with a reduction in the number and motility of sperm by as much as 25% (*Kort et al.*, 2006). Overweight is associated with reduced physical activity and long periods of sitting. In men, adipose tissue tends to grow in the abdomen and lower abdominal region. The accompanying fattening of the scrotum hinders heat transfer through the testes, mainly through an increased pressure on the testicular veins, which leads to venous stagnation (*Shafik* and *Olfat*, 1981a,b). However, the results of the studies on obesity effect influencing male fertility are ambiguous, as scrotum fat was also observed in men who were not obese (*Shafik* and *Olfat*, 1981a). Some authors also observed that the removal of excessive fat around the pubic region improved the standard seminal parameters in nearly 65% of infertile patients, and 20% of them managed to achieve pregnancy (*Shafik* and *Olfat*, 1981b).

The adverse effect of febrile episodes on semen variables, affecting male fertility has also been demonstrated. Decreasing concentration as well as loss of progressive motility and normal morphology are the most frequent alterations revealed in spermatozoa attributable to fever episodes (*Jung et al.*, 2001; *Carlsen et al.*, 2003). Interestingly, an increase of small-head spermatozoa in semen specimen collected a one-day after acute fever has also been observed (*Andrade-Rocha*, 2013). A comparative analysis of the results of standard semen analysis in a patient before a two-day fever due to influenza and on days 15, 37, 58, 79 and above 180 after fever episode showed the reduction of sperm count and sperm motility to day 58 and 37, respectively. Semen analyses performed at subsequent time points have revealed a complete return of these parameters to the initial levels before the occurrence of the fever incident (*Sergerie et al.*, 2007). In available reports on the effect of fever on semen quality, all the authors agreed that its detrimental effect on sperm parameters is transient, and the extent of observed sperm alterations depends on the height of temperature, duration of fever and stages of spermatogenic cycle in which an episode of fever occurred (*Carlsen et al.*, 2003).

Effect of environmental factors associated with raised scrotal temperature on conventional sperm parameters

Behavior and lifestyle are modifiable factors. They affect testicular exposure to temperature, may exacerbate it or help to avoid it consciously.

The temperature of the testes depends on the position of the scrotum, which changes with the position of the body. The lowest temperature can be achieved with the body straightened out, as it facilitates heat dissipation (*Zorgniotti et al.*, 1973). In comparison to sitting position, walking causes a smaller temperature increase, because due to air circulation it is possible to release heat more effectively. The temperature of the testes increases when sitting position is maintained for a long period of time (testes are placed between the thighs). Men with paraplegia, using wheelchairs, showed an increase in scrotum temperature. Moreover, an increase in the percentage of sperm with impaired motility was also observed (*Brindley*, 1982). The position of the body on the chair also affects perineum temperature; the intersection of legs generates more heat than the position with legs widened, revealing the perineum (*Koskelo et al.*, 2005; *Mieusset et al.*, 2007). However, there were no significant differences in scrotum temperature in case of lying down position; both the

group of men with paraplegia and the group of healthy men achieved similar results (*Brindley*, 1982).

The scrotum temperature increases by about 1.5–2°C even when wearing tight clothing (*Mieusset et al.*, 2007). The right choice of underwear can minimize an increase in scrotum temperature. Lower scrotum temperatures were achieved during sleeping naked than in the case of men using sleeping underwear (*Brindley*, 1982). It was demonstrated that tight underwear leads to a reduction in sperm motility (*Laven et al.*, 1988; *Lynch et al.*, 1986) and concentration (*Tiemessen et al.*, 1996). However, no significant differences between the studied groups were found in some studies comparing the effect of wearing boxer shorts adjacent to the body and loose underwear (*Munkelwitz et al.*, 1998).

In the era of universal access to electronic equipment, many people use laptops for their work. These devices emit heat. The influence of laptops placed for a few hours in the perineum area, at crossed legs, was investigated. It was demonstrated that this setting raises the temperature in the scrotum and may have a negative impact on standard sperm parameters (*Sheynkin et al.*, 2005).

Using a sauna is one of the methods of relaxation and used to cleanse the body. Studies were conducted to check an effect of a single exposure to heat in a sauna (85°C for 20 minutes) on semen parameters. It was shown that a single exposure to heat stress caused a significant decrease in sperm concentration during the first week after the exposure and normalization of the results in the fifth week after exposure (*Brown-Woodman et al.*, 1984). Studies monitoring the scrotum temperature during exposure to a sauna (87.6 ±1.3°C, humidity <15%) showed that it reaches the standard body temperature after about 10 minutes of exposure (*Jockenhovel et al.*, 1990). Two-week exposure to a sauna (80–90°C for 15 minutes) for a period of 3 months (length of seminiferous epithelium cycle) in men with normozoospermia showed a significant reduction in sperm concentration and progressive motility (*Garolla et al.*, 2013). Subsequent studies also confirmed a change in mitochondrial function and abnormal sperm chromatin protamination in men who regularly used a sauna (*Garolla et al.*, 2013).

Sperm quality can be affected by the seasons of the year and the accompanying temperature changes. Differences in the number of spermatozoa, depending on the season, were found in men living in different European cities. The values obtained in summer were 30% lower than in winter. There were, however, no significant differences in sperm motility and morphology (*Jørgensen et al.*, 2001). Interestingly, healthy Australians did not show any correlation between the season of the year and sperm concentration in ejaculate (*Mallidis et al.*, 1991).

An increased temperature of the scrotum may accompany certain occupations. This is due to specific body position or exposure to external heat. Men who work as welders are exposed to high, long-acting temperatures,

Table 1. Conventional semen parameters in men exposed to external or internal hyperthermia

Thermogenic factor	Sperm parameter	References
sauna	↓ concentration	*Brown-Woodman et al.*, 1984; *Garolla et al.*, 2013
	↓ motility	*Brown-Woodman et al.*, 1984; *Garolla et al.*, 2013
	↓ velocity	*Saikhun et al.*, 1998
welders	↓ concentration	*Bonde*, 1990; *Kumar et al.*, 2003
	↓ motility	*Bonde*, 1990; *Kumar et al.*, 2003
	↓ morphology	*Kumar et al.*, 2003
	↓ hypo-osmotic swelling sperm	*Kumar et al.*, 2003
ceramic oven operations	↓ velocity	*Figa-Talamanca et al.*, 1992
professional drivers	↓ concentration	*Henderson et al.*, 1986; *Figa-Talamanca et al.*, 1996
	↓ motility	*Chia et al.*, 1994
transient scrotal hyperthermia	↓ concentration	*Rao et al.*, 2015
	↓ progressive motility	*Rao et al.*, 2015
	↓ hypo-osmotic swelling sperm	*Rao et al.*, 2015
wheelchair	↓ motility	*Brindley et al.*, 1982
obesity	↓ concentration	*Shafik et al.*, 1981a,b
	↓ motility	*Kort et al.*, 2006; *Shafik et al.*, 1981a,b
febrile episodes	↓ concentration	*Carlsen et al.*, 2003; *Jung et al.*, 2001; *Andrade-Rocha*, 2013
	↓ motility	*Carlsen et al.*, 2003; *Jung et al.*, 2001
	↓ morphology	*Carlsen et al.*, 2003; *Andrade-Rocha*, 2013
cryptorchidism	↓ concentration	*Trsinar* and *Muravec*, 2009
	↓ motility	*Trsinar* and *Muravec*, 2009
varicocele	↓ concentration	*Abd-Elmoaty et al.*, 2010; *Shiraishi et al.*, 2010
	↓ motility	*Abd-Elmoaty et al.*, 2010; *Shiraishi et al.*, 2010

Table 2. Non-conventional semen parameters, including pro- and antioxidative, apoptotic, epigenetic, and immunologic markers in men exposed to external or internal hyperthermia

Thermogenic factor	Semen parameter	References
sauna	↓ mitochondrial membrane potential	*Garolla et al.*, 2013
transient scrotal hyperthermia	↑ malondialdehyde	*Rao et al.*, 2015
	↑ DNA fragmentation	*Rao et al.*, 2016
	↓ mitochondrial membrane potential	*Rao et al.*, 2016
obesity	↑ DNA fragmentation	*Kort et al.*, 2006
varicocele	↑ malondialdehyde	*Abd-Elmoaty et al.*, 2010; *Abo El-Khair et al.*, 2017; *Micheli et al.*, 2016; *Mostafa et al.*, 2012, 2016
	↑ 8-hydroxy-2'-deoxyguanosine	*Sakamoto et al.*, 2008
	↑ reactive oxygen species	*Wang et al.*, 2015; *Saleh et al.*, 2003
	↑ nitric oxide secretion	*Abd-Elmoaty et al.*, 2010; *Sakamoto et al.*, 2008
	↓ total antioxidant capacity	*Hendin et al.*, 1999; *Saleh et al.*, 2003
	↑ superoxide dismutase	*Sakamoto et al.*, 2008
	↓ superoxide dismutase	*Abd-Elmoaty et al.*, 2010; *Mostafa et al.*, 2012
	↓ catalase	*Abd-Elmoaty et al.*, 2010; *Mostafa et al.*, 2012
	↓ glutathione peroxidase	*Abd-Elmoaty et al.*, 2010; *Mostafa et al.*, 2016
	↓ ascorbic acid	*Abd-Elmoaty et al.*, 2010
	↑ DNA fragmentation	*Blumer et al.*, 2012; *Saleh et al.*, 2003; *Smith et al.*, 2006; *Vivas-Acevedo et al.*, 2014
	↓ soluble Fas concentration	*Fujisawa and Ishikawa*, 2003
	↑ Bax protein expression	*Mostafa et al.*, 2016
	↓ Bcl2 protein expression	*Mostafa et al.*, 2016
	↓ microRNA-122	*Mostafa et al.*, 2016
	↓ microRNA-181a	*Mostafa et al.*, 2016
	↓ microRNA-34c5	*Mostafa et al.*, 2016
	↓ microRNA-15a	*Ji et al.*, 2014
	↑ interleukin 6	*Moretti et al.*, 2009; *Nallella et al.*, 2004; *Sakamoto et al.*, 2008
	↑ interleukin 17A	*Sabbaghi et al.*, 2014
	↑ interleukin 18	*Zeinali et al.*, 2017
	↑ interleukin 37	*Zeinali et al.*, 2017
	↑ tumor necrosis factor-related apoptosis-inducing ligand	*Eid and Younan*, 2015

accompanied by contact with toxic substances and inhalation of their vapors during their work. Studies carried out in a group of men with this profession showed a reversible deterioration in semen quality (*Bonde*, 1990; *Kumar et al.*, 2003). Bakers and men working with hot ceramic ovens impregnated female partners after longer period of time compared to men who were not exposed to high temperatures (*Figa-Talamanca et al.*, 1992; *Thonneau et al.*, 1997). In turn, the assessment of semen quality among workers in the steel industry indicated the reduction of semen volume, sperm count, morphology as well as sperm motility. Moreover, these parameters were significantly correlated with scrotal and oral temperature measured in workers exposed to heat (*Hamerezaee et al.*, 2018). Studies carried out in a group of professional drivers and men with long commuting time also showed that they were more often predisposed to an increased scrotum temperature and weakened standard sperm characteristics. Additionally, they demonstrated longer time to achieve the pregnancy (*Bujan et al.*, 2000; *Chia et al.*, 1994; *Figa-Talamanca et al.*, 1996; *Henderson et al.*, 1986; *Sas and Szollosi*, 1979; *Thonneau et al.*, 1996). Some sports, e.g. competitive cycling, may also affect testicular temperature, and the negative impact on male fertility depends on the length and intensity of exercise (*Jung et al.*, 2008).

A majority of the studies have shown an adverse influence of both internal and external testicular heat on sperm production, motility and morphology due to partial or complete spermatogenic arrest (Table 1). In contrast to these studies, some authors suggested that scrotal hyperthermia is not a sufficient factor to play a role for reduced sperm quality in male population (Bonde, *2002*). Regardless of these conflicting opinions, the possible pathways involved in heat-induced germ cell damage include oxidative stress response, apoptotic/necrotic processes, epigenetic modifications, and immune/autoimmune response (Table 2) (*Ahmad et al.*, 2012; *Durairajanayagam et al.*, 2015; *Shiraishi et al.*, 2010).

Oxidative stress response and hyperthermia

It is well documented that an increased production of reactive oxygen species (ROS) and/or a decrease of the antioxidant defense cause sperm abnormalities. Increased oxidative stress is associated, among others, with semen hyperviscosity, inhibition of sperm motility, decrease in sperm concentration, and/or sperm DNA integrity in infertile males (*Aitken et al.*, 2016). Long exposure to oxidative stress may be the cause of disorders in cell metabolism as a result of ROS interactions with subcellular structures (*Fraczek et al.*, 2016; *O'Flaherty and Matsushita-Fournier*, 2017). There are strong premises that imbalance between pro- and antioxidant systems may be involved in the suppression of spermatogenesis directly related to scrotal hyperthermia. This is evidenced by experimental prospective studies in which transient scrotal hyperthermia increased the level of seminal lipid peroxidation measured by malondialdehyde (MDA) level. However, these changes were not accompanied by simultaneous severe changes in the enzymatic antioxidant systems (*Rao et al.*, 2015).

Violation of the local redox balance seems to play a principal role in the development of infertility in patients with varicocele. Some authors reported significantly elevated levels of ROS in semen of patients with varicocele (*Ishikawa et al.*, 2007; *Sakamoto et al.*, 2008). Moreover, a positive correlation between the increased seminal ROS and varicocele grade levels has also been suggested (*Allamaneni et al.*, 2004). Among classic oxidative stress markers, a significant increase in MDA concentration in the seminal plasma of varicocele patients compared to the control group was often observed (*Abo El-Khair et al.*, 2017; *Micheli et al.*, 2016; *Mostafa et al.*, 2016). Taking into account the increasing number of clinical reports confirming the presence of oxidative stress in semen of men with varicocele, it is possible that this group of patients may be specially suitable for redox evaluation as additional diagnostic assay besides routine infertility work up.

Experimental studies indicate the contribution of oxidative stress to infertility in men with a history of cryptorchidism. High production of ROS in the testes of mice with copper, zinc superoxide dismutase (Cu, ZnSOD) gene knockout was demonstrated in the experimental model of cryptorchidism (*Ishii et al.*, 2005). These studies suggested that the oxidation of subcellular structures in germ cells, mainly lipids and DNA, may directly lead to apoptosis, and the production of ROS itself may indirectly activate this process. Sparse clinical studies conducted so far in the group of adult men treated in childhood for cryptorchidism seem to confirm experimental reports and also indicate the involvement of oxidative stress in the pathogenesis of DNA sperm damage in these patients (*Smith et al.*, 2007). The redox imbalance was also observed in boys born with this developmental abnormality. The study showed a significant increase in MDA levels in the blood of boys treated for cryptorchidism compared to healthy boys. Moreover, the level of MDA depended on the type of cryptorchidism and was the highest in the group of boys with abnormal localization of both testes (*Imamoğlu et al.*, 2012).

It has already been documented in clinical and experimental prospective studies that disorders of pro- and antioxidative balance in semen are related to the reduction of male fertility in men exposed to both internal and external factors. The analysis of current reports shows that there is a tendency to combine oxidative stress and sperm death processes in pathomechanism of male infertility (*Aitken et al.*, 2012; *Muratori et al.*, 2015). However, this phenomenon was not confirmed in ejaculates of men exposed to scrotal hyperthermia.

Sperm death processes and hyperthermia

The essential prerequisite for the proper course of spermatogenesis is the homeostasis within the seminiferous epithelium, and the mechanism of programmed cell death plays a key role in this process. Apoptosis is an active process that takes place in a controlled and orderly manner. Under physiological conditions it regulates the number of spermatozoa at all stages of their development and participates in germ cells elimination. On the other hand, it may be the cause of dysregulation of spermatogenesis controlling system and the final effect or response to various pathologies. The apoptotic death of male gametes and its influence on the reproductive potential of spermatozoa have been of interest to researchers for many years and are the subject of discussion, especially in the context of a novel theory of intrinsic mitochondrial-dependent apoptosis in mature spermatozoa (*Aitken et al.*, 2012).

Recently, the direct relationship between sperm apoptosis and mild induced testicular and epididymal hyperthermia has often been reported. This was evidenced by studies in which volunteers subjected to scrotal warming demonstrated an increase in the percentage of sperm with caspase 3 activity (*Zhang et al.*, 2015a,b) and DNA fragmentation (*Ahmad et al.*, 2012; *Zhang et al.*, 2015b). Additionally, these changes were accompanied by sperm chromatin condensation alterations (*Zhang et al.*, 2015a) and chromosomal abnormalities (*Zhang et al.*, 2018).

Cryptorchidism studies carried out using rodents showed an increased level of apoptosis of germ cells in testes, which was associated with an increased DNA damage, and these changes in turn were accompanied by decreased testicular weight and infertility (*Banks et al.*, 2005, *Setchell*, 1998). In another study in a mouse model, it was shown that germ cells are able to "survive" cryptorchidism, however, most of the spermatozoa exhibited abnormalities in the nuclear DNA (*Banks et al.*, 2005). An increase in sperm DNA fragmentation in cryptorchid

men has also been demonstrated. Additionally, a causative effect between sperm DNA damage and oxidative stress in these patients has been strongly suggested (*Smith et al.*, 2007). Interestingly, in electronic microscopic studies some authors observed a higher percentage of sperm with apoptosis and necrosis in semen of men with history of cryptorchidism compared to the group of fertile men (*Moretti et al.*, 2007). Analysis of histological findings of testicular biopsies obtained during orchidopexy also indicated an imbalance between apoptosis and proliferation, especially after hormonal treatment (*Dunkel et al.*, 1997) and in abdominal cryptorchidism (*Ofordeme et al.*, 2005).

The role of programmed sperm death in the pathomechanism of infertility associated with varicocele is widely postulated in available literature. In patients with this disease, an increase in phosphatidylserine translocation (*Chang et al.*, 2010; *Wu et al.*, 2009), a decrease in mitochondrial membrane potential, an increase in DNA fragmentation (*Chang et al.*, 2010; *Cortés-Gutiérrez et al.*, 2016; *Peluso et al.*, 2013; *Wu et al.*, 2009), an increase of active caspases 3/7 (*Foroozan-Broojeni et al.*, 2018), an increase in expression of p53, pro-apoptotic (Bax) and anti-apoptotic (Bcl2) proteins (*Chang et al.*, 2010) were often observed in ejaculated sperm. Increased apoptosis of germinal cells was also observed in biopsies from testicles of patients with varicocele (*Hassan et al.*, 2009). On the other hand, there are some reports in which the authors observed reduced apoptotic activity in testes of patients with varicocele (*Fujisawa et al.*, 1999). It is important to note that the existence of proapoptotic mechanisms associated with mitochondria damage of the male gametes were also strongly suggested by some authors. Varicocele has been correlated with a high percentage of sperm with inactive mitochondria, and the oxidative stress found in patients with this pathology can be additional explanation for this observation (*Dieamant et al.*, 2017; *Foroozan-Broojeni et al.*, 2018). In the light of these data the induction of mitochondrial-dependent intrinsic pathway as male gonad response to varicocele-related heat stress is strongly emphasized.

Among the classic apoptotic parameters, the greatest discussions concern the role of the assessment of sperm DNA fragmentation in men with varicocele (*Cho et al.*, 2016). In varicocele patients, an increase in the percentage of spermatozoa with DNA fragmentation in semen was documented most frequently, which was associated with the intensification of apoptosis and abnormal levels of sex hormones (*Durairajanayagam et al.*, 2015; *Ku et al.*, 2005). These disorders were usually accompanied by oligo-, astheno- and/or teratozoospermia (*Nieschlag et al.*, 2010; *Park et al.*, 2018). In men with varicocele, disorders of sperm DNA integrity seem to be critical for the biological dysfunction of spermatozoa. In this context, further research on the diagnostic and prognostic significance of an evaluation of sperm nuclear DNA in infertile men with this pathology, especially in terms of qualification for surgical removal of varicocele, is fully justified (*Esteves et al.*, 2017).

Epigenetic modifications and hyperthermia

The 'poor' sperm DNA quality is one of the undisputable factors affecting male reproductive ability both in natural and assisted procreation. Analysis of sperm DNA quality cannot be conducted in isolation from the studies on sperm epigenetics which may be a breakthrough in the field of male reproduction with respect to infertility as well as embryonic development (*Carrell*, 2012; *Denomme et al.*, 2017). There is growing evidence that male infertility might be associated with the epigenetic status of human sperm characterized by DNA methylation level, specific modifications of retained histones, and non-coding microRNAs (miRNAs) expression (*Cui et al.*, 2015; *Laqqan et al.*, 2017; *Schon et al.*, 2018).

It is well accepted that stress conditions (e.g. hyperthermia, oxidative stress) alter the biogenesis of miRNAs and gene expression (*Leung and Sharp*, 2010; *Wilmink et al.*, 2010). The role of miRNAs in spermatogenic impairment associated with heat stress has also been indicated. Next-generation sequencing-based miRNAs profiling of mice testis subjected to transient hyperthermia revealed detailed miRNAs profile critical to heat stress-induced testicular damage (*Rao et al.*, 2017). Additionally, the authors suggested that target genes of these miRNAs may be involved in germ cell apoptosis pathways. Similar to the observations in experimental heat stress conditions, the negative correlations between a few miRNAs and seminal markers of apoptosis as well as oxidative stress in seminal plasma of infertile patients with varicocele were also observed (*Mostafa et al.*, 2016). Further, the analysis of miRNAs expression in spermatozoa indicated significant downregulation of microRNA-15a (miR-15a) in patients with varicocele compared to fertile controls (*Ji et al.*, 2014). Moreover, miR-15a repressed the expression of heat shock protein family A (Hsp70) member 1B (*HSPA1B*) gene, coding a typical heat shock chaperon protein. These interesting data suggested that the decreased expression of some miRNAs may be one of the mechanisms that contribute to the protection of heat stress-induced damage in spermatozoa.

Out of epigenetic regulators, sperm DNA methylation raises the most controversies and many questions still remain unresolved. For example, it is unknown whether the production of defective spermatozoa is associated with a global hypo- or hypermethylation of DNA, or which environmental agents can be responsible for epigenetic modification of sperm DNA. The potential links between aberrant sperm DNA methylation and impaired sperm DNA integrity have been extensively discussed in recent papers. An association between oxidative stress and the global methylation status of the

sperm genome has also been suggested (*Olszewska et al.*, 2017). Another new interesting finding reveals that the tendency of spermatozoa to enter intrinsic apoptotic cascade can be associated with disorders of spermatogenesis due to a global hypermethylation of nuclear DNA (*Barzideh et al.*, 2013). The first premises for the involvement of methylation disorders in male infertility related to varicocele have been recently published (*Tavalaee et al.*, 2015). The authors postulated that individuals with varicocele showed increased DNA susceptibility to damage when DNA was hypomethylated, and this phenomenon appears to be independent of ROS production. With respect to cryptorchidism, to date no human studies considering the importance of sperm DNA methylation in this pathology have been published. However, in animal model, sperm genomic methylation changes appear to be a risk factor in the development of cryptorchidism across generations (*Chen et al.*, 2015).

Immune/autoimmune response and hyperthermia

The impairment of sperm production and function can be related to immunological status of spermatozoa local environment. The role of hyperthermia may also be considered in this aspect.

Antisperm antibodies (AsA) are the most frequent biomarkers of immunological infertility. They can cause infertility blocking different phases of fertilization process, and their formation is usually associated with the disruption of the blood-testis barrier or the deficiency of immunosuppressive agents that play a role in maintaining an active tolerance to male gametes. In normal circumstances spermatozoa do not trigger an immune response. Due to disturbances in immunoregulatory mechanisms of testes, AsA are produced as a consequence of sperm exposure to the immune system (*Chiu et al.*, 2004; *Restrepo* and *Cardona-Maya*, 2013). Several theories have been created to explain the mechanism by which AsA may impair male infertility. However, the effect of exposure to environmental pollution (out of occupation) such as heat, radiation, sound or vibration on the formation of AsA has not been studied (*Tennakoon*, 2013). The incidence of AsA is associated with epididymal or testicular failures induced by the testicular/scrotal hyperthermia, e.g. testicular trauma, torsion, cryptorchidism, varicocele, mobile testis/es, epididymitis, prostatitis. Some authors consider hyperthermia as a possible cause of AsA production in varicocele (*Walsh and Turek*, 2009). In turn, other authors observed the reduction of AsA levels after varicocelectomy (*Djaladat et al.*, 2006). However, in current research, there is suggested rather the lack of an association between varicocele (*Veräjänkorva et al.*, 2003) or cryptorchidism (*Jiang* and *Zhu*, 2013) with immune related infertility. Whenever or not a varicocele or cryptorchidism lead to development of AsA still remains controversial. It is not clarified why in patients with varicocele, AsA are revealed only in 30%. Some authors suggested that varicocele is not an immediate cause of autoimmune reactions against spermatozoa but is a cofactor increasing AsA development (*Bozhedomov et al.*, 2015). We have to remember that AsA formation is the end point of a complex immunological process. A recent study by *Lotti et al.* (2018) supports that AsA formation does not depend just on direct testicular injury but it is rather due to an epididymal inflammation, which might extend to the testicular interstitium inducing a compensated Leydig cells impairment but not tubular damage.

The mechanism by which fertility may be affected by AsA is multifactorial and complicated, and the reason remains controversial. The pathogenesis of sperm dysfunction in immune infertility can be associated with the oxidative stress of spermatozoa (*Bozhedomov et al.*, 2015). Reactive oxygen species production in AsA positive varicocele patients was higher than in AsA negative varicocele patients and fertile men (*Bozhedomov et al.*, 2014). Cryptorchidism may also present a causative link with oxidative stress (*Tremellen*, 2008). Prolonged testicular/scrotal hyperthermia in clinical conditions (e.g. varicocele or cryptorchidism) may induce oxidative stress response and trigger the immune-based reaction. Another indirect mechanism of AsA action is to mediate the release of cytokines and thus to induce sperm cytotoxity, increase sperm phagocytosis and impair sperm function.

Cytokines are soluble mediators of immune cell function produced by lymphoid and non lymphoid cells playing a key role in the afferent and efferent phases of immune responses of both the innate and acquired immunity (*Perdichizzi et al.*, 2007). Cytokines are produced physiologically in the male gonads and are involved in normal testicular function. They are generated by germ cells, testicular macrophages, Leydig, Sertoli, immune and mesenchymal/myeloid cells. Cytokines are very important in the establishment and maintenance of immune-privilege of the testis. In normal physiological conditions, anti-inflammatory cytokines like interleukin (IL)-10, IL-13, IL-14, transforming growth factor beta (TGF-β) are released for creating tolerance against sperm cells (*Loveland et al.*, 2017). The secretion of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, tumor necrosis factor alpha (TNF-α) is one of the first signals from the innate host defense to combat genital tract inflammation/infection (*Fraczek* and *Kurpisz*, 2015). Elevated cytokine levels in seminal plasma negatively influence sperm parameters. Of the various cytokines, IL-6 was the most frequently found in high levels in patients with clinical factor of hyperthermia such as varicocele (*Nallella et al.*, 2004; *Moretti et al.*, 2009, 2014; *Sakamoto et al.*, 2008) and cryptorchidism (*Imamoğlu et al.*, 2012). According to some authors, varicocele-related infertility can also be connected with overexpression of seminal IL-17A or TNF-related apoptosis-inducing ligand (TRAIL)

(*Eid* and *Younan*, 2015; *Sabbaghi et al.*, 2014). In turn, in experimentally-induced varicocele in animal model, an increase in IL-1α and IL-1β in testes was observed (*Sahin et al.*, 2006).

Different cytokines stimulate a diverse response of cells involved in immunity and inflammation. Cell-mediated immunity is undoubtedly related to ROS elevation and the activation of proteases as well as cytokines release during inflammatory reactions (*Aitken* and *Baker*, 2013). Among various cytokines, seminal IL-8 is regarded as a reliable surrogate marker of male urogenital tract inflammation. Moreover, a relationship between AsA level and IL-8 concentration has been suggested (*Lotti* and *Maggi*, 2013). The impact of acute genital tract infections on fertility is widely accepted but silent genital inflammation not related to semen cultures is poorly understood (*Eggert-Kruse et al.*, 2007). Inflammation plays a role in the pathophysiology of varicocele. Significantly increased levels of pro-inflammatory IL-18 and anti-inflammatory IL-37 were observed in infertile men with this pathology. The interaction between IL-37 and IL-18 receptor can lead to reduced inflammatory responses, and IL-37 might be a potential biomarker for male infertility (*Zeinali et al.*, 2017). Another inflammatory marker such as mean platelet volume was also significantly higher in subfertile patients with varicocele compared to fertile men (*Demirer et al.*, 2018). To sum up, cytokines are an essential part of the inflammatory effect caused by varicocele. Moreover, these bioactive substances may constitute an important link between varicocele and infertility. Pathological mechanisms such as oxidative stress and apoptosis appear to be the main factors of the cytokine-mediated testicular dysfunction but there is a need for more prospective studies to prove the involvement of hyperthermia in these processes.

Contraceptive use of hyperthermia

Controlled hyperthermia has been a matter of application for a long time beginning from the 1950s (India) when *Voegeli* (1956) proposed hot sitting baths (47°C for 45 minutes daily for 3 weeks) to induce reversible male infertility for six months. Neither prospective nor retrospective data have been published. However, an exact temperature survey was followed indicating scrotal temperature of 40.5°C (median) when water temperature was at the level of 43°C. Semen analyses have not been performed within this survey. An interesting cycle of experiments was demonstrated by *Watanabe* (1959) when scrotum has been immersed into hot water bath and a single exposure for 30 min in approx. 45°C was applied to 18 volunteers. After 5–8 weeks a decrease in semen quality was observed, however, in 2–5 volunteers only. When repeating the procedure daily for up to 12 days, a compromise in semen quality was observed for 5–12 weeks with return to normal levels thereafter. So, a reversible effect was demonstrated. In another study, oligozoospermic males (n = 20) responded well to a regime of scrotal heating in a water bath with 43–45°C for 30 minutes on six alternate days. Reversible impairment in sperm quality was noticed between 11–112 days post exposure (*Rock* and *Robinson*, 1965). The next study was conducted by using the body itself as a source of genital heat. Induction of heat stress was induced by three approaches: a) fixing the scrotum close to the inguinal canal (n = 15 volunteers, b) wearing a suspensor pressing the testes to the inguinal canal (n = 13), c) wearing an insulating polyester suspensory elevating towards abdominal wall (n = 14). As a result, in 33 men azoospermia was developed (*Shafik*, 1991, 1992). A more advanced study was performed by *Wang et al.* (1997) in which 21 normozoospermic volunteers were subjected to wearing polyester-lined supports elevating the testes to the abdominal wall (52 weeks for 23 h daily). However, the increase of 1°C in scrotal temperature did not bring a decrease in semen quality.

The relatively recent approach instrumented by *Jia et al.* (2007) used nonhuman primates (cynomolgus monkeys) applying mild testicular hyperthermia (43°C for 30 min for two consecutive days), hormonal deprivation (testosterone) or both. Subsequently testicular biopsies have been performed after treatment. It has been disclosed that treatment with testosterone, heat or combined led to activation of mitogen-activated protein kinases (MAPKs) including MAPK 1/3 and MAPK 14 accompanied by an increase of B-cell leukemia/lymphoma 2 (Bcl2) in both cytosolic and mitochondrial fractions of testicular lysate as well as cytochrome c and second mitochondria-derived activator of caspases (DIABLO) release. Inactivation of Bcl2 was achieved through phosphorylation at serine 70 thus favoring the mitochondria-dependent death pathway. Specifically DIABLO released from mitochondria into cytosol promotes apoptosis by antagonizing inhibitor of apoptosis proteins (IAPs). The other potentially important pathway (extrinsic) which involves ligation of the Fas cell surface death receptor (FAS) could also potentially enhance apoptotic process, however, its role in hyperthermia has been unproven. More information about molecular mechanism associated with the reversible suppression of spermatogenesis induced by heat administration came from research of global proteomic analyses of the human testis. *Zhu et al.* (2010) identified the changed expression of series of 26 known proteins taking part in the complex functional network. Most of them were involved in the events related to promotion of apoptosis and suppression of proliferation as well as cell survival. Out of the proteins, heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1) was found to be an important anti-apoptosis protein that could regulate the expression of other heat-induced proteins, and it seems to be the potential target for contraceptive development. Despite the progress of knowledge about the mechanisms mediating the effect of transient hyperthermia

on blocking spermatogenesis, further studies are needed to design contraceptive methods based on heat stress.

Final remarks

The effect of testes overheating on male fertility has not yet been fully understood. Numerous studies regarding testicular/scrotal hyperthermia have been carried out in animal models, but the underlying mechanism for reduced fertility as a consequence of genital heat stress still remains a puzzle. The main reason for this situation is a relatively small number of controlled prospective studies with regard to various heat stress as well as a wide range of analyzed seminal parameters. However, hyperthermia should be seriously considered as an important factor responsible for transient and/or persistent infertility risk factor and actions towards removal of genital heat stress shall be pursued in male infertile patients. Specific work-up for temperature-dependent contraceptive method has not been yet reliably elaborated although it could be a relatively harmless and reversible procedure.

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