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Experimental mild increase in testicular temperature has drastic, but reversible, effect on sperm aneuploidy in men: A pilot study

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ABSTRACT

In mammals testicular and epididymal temperature increase impairs spermatogenesis. This experimental study investigates the effects of a mild testis temperature increase (i.e. testis temperature remains below core body temperature) on sperm aneuploidy in men. In 5 fertile volunteers a testicular temperature increase was induced by maintaining the testes at suprascrotal position using specially designed underwear for 15 ± 1 h daily for 120 consecutive days. After heating men were followed for next 180 days. A control group (27 men) was recruited. Semen samples were collected before, during and after heating period and analyzed for chromosomes X, Y and 18 for aneuploidy using FISH. A total of 234,038 spermatozoa were studied by FISH. At day 34 of heating, mean sperm aneuploidy values were not modified. From day 34 of heating until day 45 post heating, FISH evaluation was not possible due to the drastic fall of sperm count. At day 45 post-heating total sperm aneuploidy percentage was twice higher than before heating whereas. Sex disomy (sperm XY18), sex chromosome nullisomy (sperm 18) were significantly higher than controls. These effects were completely reversed at 180 days post heat exposure. Conclusion: A mild rise in testicular temperature significantly increases sperm aneuploidies, reflecting an effect on the meiosis stage of spermatogenesis. The effect of heating was reversible and suggests that recovery of aneuploidy to normal values requires at least two cycles of spermatogenesis. Nonetheless, the low number of volunteers was a limitation of this pilot study and warrants further research on larger population.

1. Introduction

The adverse effects of increased testicular temperature on spermatogenesis in mammals have been investigated and established for many years. In most mammals, testes are located in the scrotum outside the body, which provides anatomical location that offers lower testicular temperature than core body. In a thermoneutral environment, the scrotal testes are appreciably cooler than the body, but this does not mean that they are kept at a constantly lower temperature [1]. In man as in most other mammals, optimal spermatogenesis requires 2–6 °C lower testicular temperature than body and any rise above the physiological temperature of the testes has adverse impact on spermatogenesis [2,3].

Several pathological conditions are associated with an increase in testicular temperature which include cryptorchidism [4], varicocele [5–7] and febrile diseases [8,9]. Exogenous factors such as occupational exposure to high temperatures [10,11], car driving [12] or sauna exposure [13–15] also increase the testicular temperature. Notably, some living conditions such as wearing tight clothing [16] or sitting and sleeping postures [17] are also associated with an increase in testicular temperature which remains in the physiological range [18]. The relation between lifestyle factors or exposure to environmental risk factors and sperm aneuploidy was investigated in several studies [19–22], but to our knowledge, only one epidemiological study has reported the possible link between circumstances known to improve testicular temperature, such as sauna use or type of underwear, and sperm

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aneuploidy and have shown decrease in sperm aneuploidy in men wearing boxer shorts compared to those wearing tight underwear [23].

In man, different experimentally methods increasing testicular temperature resulted in a drastic, but reversible, inhibition of spermatogenesis with decrease in sperm count [13,24–28] sperm motility [13,24–27] and sperm normal morphology [13,25,26]. In addition to conventional sperm parameters (count, motility, morphology) the adverse effects of testicular hyperthermia on sperm DNA damage have also been reported [13,24].

It has been suggested that a modification of testicular temperature could be used in two ways: increasing testes temperature to inhibit spermatogenesis or decreasing it to improve spermatogenesis in infertile men who have abnormally elevated testes temperatures. Experimental studies [29–31] and reviews [32–34] have reported that a mild testis temperature increase could be used as a male contraceptive approach. On the other hand, reducing the testicular temperature using application of an ice bag for 30 min per day for 14 days improves spermatogenesis outcome [35]. Various devices have been used to decrease testicular temperature and improve spermatogenesis, either in varicocele or in oligozoospermic patients having higher scrotal temperatures [36–38]. In addition, avoidance of exposure to heat has been reported to prevent infertility [39–41].

We have previously demonstrated that a mild increase in testicular and epididymal temperature (+2 °C, i.e. a testicular temperature below core body temperature), induced by maintaining the testes in a suprascrotal position, had drastic effects not only on sperm output and quality but also on sperm DNA fragmentation in humans [24].

While a study in mice [42] reported X-Y dissociation increase in spermatocytes from animals that were exposed to an environmental temperature of 35 °C for 2–5 days (whole body exposure), to date no study has investigated the effect of a mild testicular temperature increase (testicular temperature below core temperature) on sperm aneuploidy in men. A recent study in man reported increased sperm aneuploidy after testicular exposure to 43 °C for two successive days per week for 3 months. [43]. Noteworthy, 43 °C is very high temperature compared to physiological temperature of testes (≈34.5 °C) and sperm aneuploidy is more likely to occur at this higher temperature threshold.

Sperm aneuploidy can be a risk to the embryonic and fetal development and the offspring [44,45]. The majority of the aneuploidy embryos are not viable resulting into miscarriages but several chromosomal abnormalities, such as autosomal trisomy or sex chromosome aneuploidy, could be non-lethal and compatible with survival resulting in birth and developmental defects [44–47].

In this context, the objective of this study was to evaluate the effects of a mild testicular temperature increase (+2 °C) on sperm aneuploidy in fertile men and to examine its potential reversibility.

2. Materials and methods

2.1. Study design

The present study is the second part of a study of the effects of mild induced testes temperature increase on gamete quality [24]. We used the biological specimens that had been frozen during the first part of the study.

2.2. Study population

The study was approved by the Ethics Committee (Comité de Protection des Personnes Sud-Ouest et Outre Mer I) and the protocol has been previously described [24]. The volunteers were recruited through advertisement in the press and local hospital communication portal. A total of 34 men volunteered for the study. After exclusion criteria and financial limitations 6 volunteers (aged 25–35 years) were selected. Only the men who had fathered at least one child, had a normal clinical andrological examination, no current pathology, medical or surgical history, had either no, moderate or occasional alcohol intake were included. None of the participating volunteer was exposed to toxic agents and had no particular professional risk to fertility. One volunteer smoke 20 cigarettes a day in experimental group and 7 men smoke more than 10 cigarettes a day in control group.

One volunteer dropped the study at day 73 of heating due to personal reasons hence the data of this volunteer were excluded. Remaining five volunteers followed the instructions and the protocol and continued until the completion of study. None of the volunteer reported any discomfort during the entire period of study. The aneuploidy results of 27 fertile men were used as control values to compare with aneuploidy values of experimental volunteers (n = 5).

2.3. Technique for increasing testicular temperature

Increased testicular and epididymal temperature was induced by maintaining the testes in a suprascrotal position by means of specially designed underwear worn 15 ± 1 h daily [3,24,29] for 120 consecutive days. The method was developed by our group and has been tested safe, reversible and reproducible. Before, commencement of heating participating volunteers of experimental group were trained on how to put the underwear by lifting the testes up at the root of the penis during waking hours. The support to the testes keeping them at suprascrotal position was ensured with the underwear provided with an orifice allowing the scrotum and penis to be exteriorized specifically designed for each volunteer as we previously described [25]. The suprascrotal position of the testes results in 1.5–2 °C increase in testicular temperature. Notably, the testicular temperature remains below core human body temperature in this heating method [30,48].

2.4. Semen analysis

Semen samples were collected, after mean sexual abstinence of 4.1 (± 1.0) days, at specific times according to spermatogenesis chronology as we previously described [19]. After liquefaction at 37 °C for minimum of 30 min, semen analysis was done according to WHO guidelines [49] on an aliquot (200 µL) by a trained technician. The characteristics considered were sexual abstinence (days), ejaculate volume (ml), sperm concentration (SC, 10^6 spermatozoa/ml) and total sperm count (volume × SC = TSC, 10^6 spermatozoa/ejaculate). Total sperm count was the only sperm parameter presented, as it is the true reflect of sperm production.

After initial analysis a part of each sample was frozen in liquid nitrogen in the Germethèque Biobank (BB-0033-00081, France) using routine freezing method currently practiced in our laboratory [50] and stored until FISH analysis.

2.5. Sperm Fluorescence In Situ Hybridization (FISH)

FISH analysis was performed at four time points i.e. before heating at D0, D34, post heating at PH45 and PH180 (Fig. 1). Between day 34 of heating and day 45 post heating total sperm count was drastically reduced due to heating method and did not recover sufficiently until day 45 post heating which rendered the FISH analysis impossible (Fig. 1). Frozen cells were thawed and washed twice with 5 ml phosphate buffer saline (PBS) and centrifugation performed at 630*g* for 10 min. Samples were then fixed with fixation solution (acetic acid and methyl alcohol) for 30 min at 4 °C. After centrifugation at 1500*g* for 10 min, the supernatant was discarded and the pellet re-suspended. A volume of 10 µl was dropped on a slide and cell density was verified by microscopy and adjusted accordingly. Slides were incubated for a minimum of 2 h at −20 °C. DNA was decondensed by incubating slides in 1 M NaOH for 1 min, washed twice in saline sodium citrate (SSC) buffer, then dehydrated in 70%, 90% and 100% ethyl alcohol baths (2 min each). Each slide was then incubated overnight with the different probes at 37 °C (Vysis probes (Abbott), CEP X spectrum green, CEP Y spectrum orange

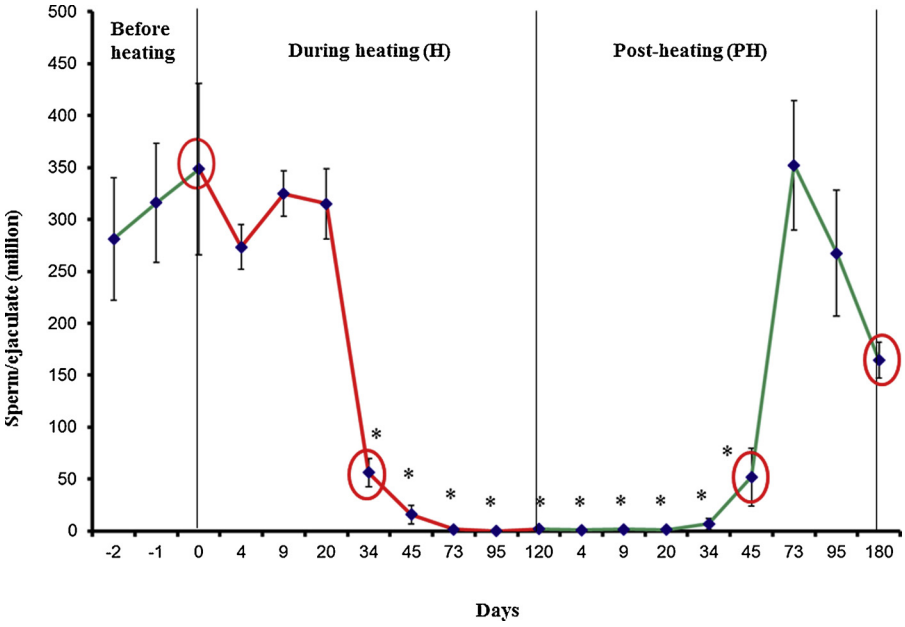

Fig. 1. Mean (SEM) total sperm count (millions per ejaculate) before, during and after a mild induced testicular and epididymal temperature increase in healthy men. Red circles: semen probes used for sperm aneuploidy study: before heating (day 0), during heating (day 34) and during post heating period (day 45 and day 180). *p < 0.016.

Table 1
Sperm aneuploidies (%) in controls and before (Day 0), during (H34) and after (PH45, 180) testicular and epididymidal heating in healthy men.

	Controls (n = 27) median [Q1–Q3]	Day 0 (n = 4) median [Q1–Q3]	H 34 (n = 5) median [Q1–Q3]	PH 45 (n = 4) median [Q1–Q3]	PH 180 (n = 5) median [Q1–Q3]
Number of spermatozoa counted per subject	5231 [5092–5335]	5082.5 [5048.5–5191.5]	5085 [5083–5143]	5076 [5047–5089.5]	5117 [5047–5120]
Y 18	49.43 [47.67–50.6]	49.96 [49.16–50.55]	50.39 [49.78–51.34]	49.15 [48.47–50.32]	48.89 [48.21–49.64]
X 18	49.82 [48.85–51.3]	49.44 [48.72–49.8]	48.98 [47.92–49.09]	48.97 [47.93–49.47]	49.66 [49.28–51.23]
XX 18	0.06 [0.04–0.06]	0.04 [0.01–0.06]	0.02 [0.02–0.04]	0.03 [0.02–0.04]	0.02 [0.02–0.02]
YY 18	0.04 [0–0.06]	0 [0–0.04]	0.02 [0–0.04]	0.04 [0.03–0.06]	0.02 [0–0.02]
XY 18	0.21 [0.13–0.31]	0.35 [0.21–0.72]	0.34 [0.32–0.49]	0.85 [0.68–1.03] (p = 0.001)*	0.18 [0.16–0.8]
Sex disomy	0.30 [0.19–0.38]	0.35 [0.24–0.80]	0.45 [0.35–0.53]	0.93 [0.76–1.10] (p = 0.001)*	0.22 [0.18–1.04]
X 1818	0 [0–0.02]	0 [0–0.02]	0 [0–0.02]	0.01 [0–0.02]	0.02 [0–0.04]
Y 1818	0.02 [0–0.04]	0.01 [0–0.03]	0.02 [0–0.02]	0.04 [0.04–0.05]	0.02 [0–0.02]
Disomy 18	0.04 [0–0.06]	0.01 [0–0.05]	0.02 [0.02–0.03]	0.05 [0.04–0.07]	0.02 [0.02–0.04]
XX 1818	0 [0–0]	0 [0–0]	0 [0–0]	0 [0–0]	0 [0–0.02] (p = 0.001)*
YY 1818	0 [0–0]	0 [0–0]	0 [0–0]	0 [0–0.01]	0 [0–0]
XY 1818	0 [0–0]	0 [0–0.03]	0 [0–0.02] (p = 0.010)*	0.01 [0–0.03] (p = 0.003)*	0 [0–0.02] (p = 0.010)*
Diploidy	0 [0–0]	0 [0–0.03]	0 [0–0.02]	0.02 [0–0.04]	0 [0–0.08]
XXY 18	0 [0–0]	0.01 [0–0.02] (p = 0.003)*	0 [0–0]	0 [0–0.01]	0 [0–0]
YYX 18	0 [0–0]	0 [0–0]	0 [0–0]	0 [0–0.01]	0 [0–0]
18	0.24 [0.17–0.33]	0.29 [0.23–0.37]	0.26 [0.25–0.31]	0.91 [0.76–0.99] (p = 0.001)*	0.41 [0.34–0.46]
X	0.06 [0–0.23]	0.01 [0–0.03]	0 [0–0]	0 [0–0.03]	0 [0–0.04]
Y	0 [0–0]	0 [0–0]	0 [0–0]	0 [0–0]	0 [0–0]
Nullisomy 18	0.06 [0–0.23]	0.01 [0–0.03]	0 [0–0]	0 [0–0.03]	0 [0–0.06]
Total aneuploidy	0.65 [0.46–0.81]	0.73 [0.58–1.19]	0.73 [0.64–0.87]	1.93 [1.62–2.19] (p = 0.002)*	0.7 [0.67–1.84]
Meiosis I	0.28 [0.17–0.32]	0.35 [0.21–0.79]	0.37 [0.34–0.51]	0.91 [0.74–1.11] (p = 0.001)*	0.20 [0.20–1.19]
Meiosis II	0.08[0.06–0.11]	0.04 [0.01–0.10]	0.039 [0.038–0.039]	0.08 [0.07–0.09]	0.04 [0.02–0.08]

Values are expressed as median with interquartile range [q1-q3]. Day 0: before heating, H: heating period, PH: post heating period. Sex disomy: sum of XX18, YY18, XY18. Disomy 18: sum of X1818 and Y1818. Diploidy: sum of XX1818, YY1818 and XY1818. Nullisomy 18: sum of X and Y. Meiosis I: sum of frequencies of XY18 and XY1818. Meiosis II: sum of XX18, YY18, XX1818 and YY18.

* p < 0.0125 between controls and volunteers at time 0, H34, PH 45 and PH180.

and CEP 18 spectrum aqua). After a 2 min wash in 2SSC 0.4% NP40 at 73 °C followed by a 1 min wash in 2SSC 03% NP40, slides were incubated with 1/2000e Hoechst for 3 min and washed for 3 min in PBS. Slides were finally mounted with Antifade mounting medium (Promega, Germany) and stocked at −20 °C until reading. Slides were analyzed under a Leica DM 6000 B microscope system.

At each time, a mean of 5149 ± 218 and 5232 ± 190 cells, per subject, were read respectively in experimental group and control group.

2.6. Statistical analysis

Data are presented in the Tables as median and interquartile range Q1–Q3 due to the number of patients and as mean and standard

deviation in graphic representations.

Sperm aneuploidy data were compared before, during and after mild induced increase in testicular and epididymal temperature by the Wilcoxon signed rank- sum test. Due to the small number of men included in the experimental protocol, we also carried out statistical comparison with a control group of 27 fertile men (a single evaluation of sperm aneuploidy for each man) using the non-parametric Mann-Whitney test. As there were multiple comparisons, a Bonferroni correction was used. P-values of 1.66% were considered significant for the Wilcoxon test and of 1.25% for the Mann-Whitney test.

Statistical analyses were performed using SAS software (9.3, SAS Institute). A p- value of 5% was considered significant without Bonferroni correction.

3. Results

The number of volunteers (n = 5) was constant during the three study periods (before, during and after heating). Due to technical problems, at D0 and PH45 four volunteers underwent FISH analyses instead of five.

Total sperm count decreased significantly as early as day 34 during heat exposure (Fig. 1) and remained persistently low (azoospermia or severe oligospermia) throughout the heating period (days 0–120). FISH analysis was not possible until day 45 after cessation of heating (PH45).

A total of 234,038 spermatozoa were analyzed by FISH. Before commencement of experimental heating (Day 0), no significant differences were found for aneuploidy percentages between the control group (n = 27) and the five volunteers except for median frequencies of XXY18. However, the range of frequencies difference was very small, ranging from 0.00 to 0.02 (Table 1).

Table 1 represents sperm aneuploidy percentages at the different time points of the study. The major significant results were an increased total aneuploidy, sex disomy (sperm XY18) and sex chromosome nullisomy (sperm 18) at day 45 post heating (PH45). The percentages of sperm with sex disomy and sex nullisomy were two and three times more elevated, respectively, than before heating period (D0). Percentage of total aneuploidy was twofold higher at PH45 than before heating (D0) (Table 1, Fig. 2).

At day 180 post heating (PH180), sperm aneuploidy percentages did not differ from the values before heating (Fig. 2), which suggests that the effects of heating were reversible.

In this study, we defined an abnormal aneuploidy rate as a rate above the 90th percentile of control group values. This enabled us to calculate the number of volunteers who had an abnormal aneuploidy rate at different time points.

The results indicate that at 45 post heating, all volunteers had a higher number of abnormal sperm with sex disomy XY18, sex nullisomy and total sperm aneuploidy (Fig. 3) which did not recover to values before heating period until day 180 post heating suggesting that minimum of two cycles of spermatogenesis and epididymal transit after discontinuation of heating are required in order to restore normal spermatogenesis with no risk of aneuploidy (Fig. 3).

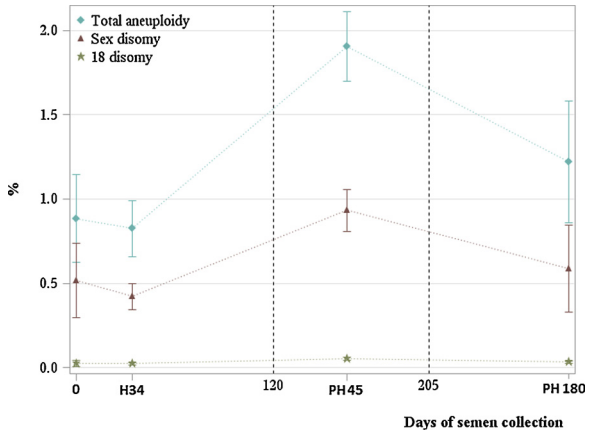

Fig. 2. Sperm aneuploidies (%) before, during and after a mild increase in testicular and epididymal temperature in men. Sperm chromosomal abnormalities (%) measured at the four time points: before heating (D0), during heating (H34), and after heating (PH45 and PH180). Means ± SEM are represented. Mild induced testicular and epididymal heating was discontinued at D120.

4. Discussion

The negative impact on spermatogenesis after testicular exposure to a mild or high temperatures increase has been demonstrated by numerous experimental human and animal studies. A recent study reported an increases sperm aneuploidy rate when testes were exposed to 43 °C [43]. However, these findings were taken at one-point time and reversibility was not evaluated. Moreover, 43 °C is a pathological temperature which is much higher than testes and core body temperature and sperm aneuploidies are more likely to occur at this high threshold. No study has investigated the effect of a mild increase in testicular temperature (< 37 °C) on the frequency of sperm chromosomal abnormalities.

In present study by inducing a mild increase in testes and epididymal temperature for 15 ± 1 h daily for 120 consecutive days, we show that this temperature threshold is sufficient to increase sperm aneuploidy in men and that this effect is reversible after cessation of heating which suggests that testicular heating is the cause of sperm aneuploidy.

At day 34 of heating, the percentage of chromosomal abnormalities were not affected by the mild temperature increase. In accordance with the physiology of the spermatogenesis process and epididymal transit timing, spermatozoa collected at D34 were at the late spermatocyte stage (end of meiosis) or early round spermatids in the testes when heat was induced: i.e. they were subjected to heat but were released from progenitor germ cells that were not exposed to heating. This is in agreement with previous reports indicating that most chromosomal abnormalities in sperm result from meiotic errors during the spermatogenesis process [51,52].

At day 45 post heating (PH45) we found a significant increase of disomic sperm (XY18 sperm: non-disjunction of the sexual chromosome at meiosis I) or nullisomic sperm (18) and a slight increase of diploidy sperm (XY1818), consequently total aneuploidy increased at this time. It is noteworthy that spermatozoa collected at day 45 after heating were more probably affected by heating at the meiosis stage, according to spermatogenesis and epididymis physiology as previously published [24].

The effects of a mild increase in testes temperature are reversible, as indicated by recovery of aneuploidy values to pre-heating values at PH180. The time require to recover the aneuploidy to control values suggest that reversibility requires more than two cycles of spermatogenesis.

A recently published study examined sperm aneuploidy in 10 men before and during scrotal heating using an electric warming bag to increase the testes temperature to 43 °C for 30–40 min two days per week for 3 months [43]. Compared to pre-heating values, a significant increase in the sum rate of sperm chromosomal 13, 18, 21, and sum of X and Y sperm was reported at the end of the 3 months of heating. In this last study, the increase of sum rate of aneuploidy for X and Y at the end of the 3-month period of heating was 14.2-fold higher than before heating values. This is much higher than the 2 to 3-fold increase we observed in the present study where temperature increase was very mild. However, the aneuploidy values after cessation of heating were not reported in the study by Zhang et al. [43] which lacks information about the reversibility. Further, only 200 sperm cells were analyzed by Zhang et al. [43] compared to present study which report analysis of 5149 ± 218 and 5232 ± 190 cells in experimental volunteers and

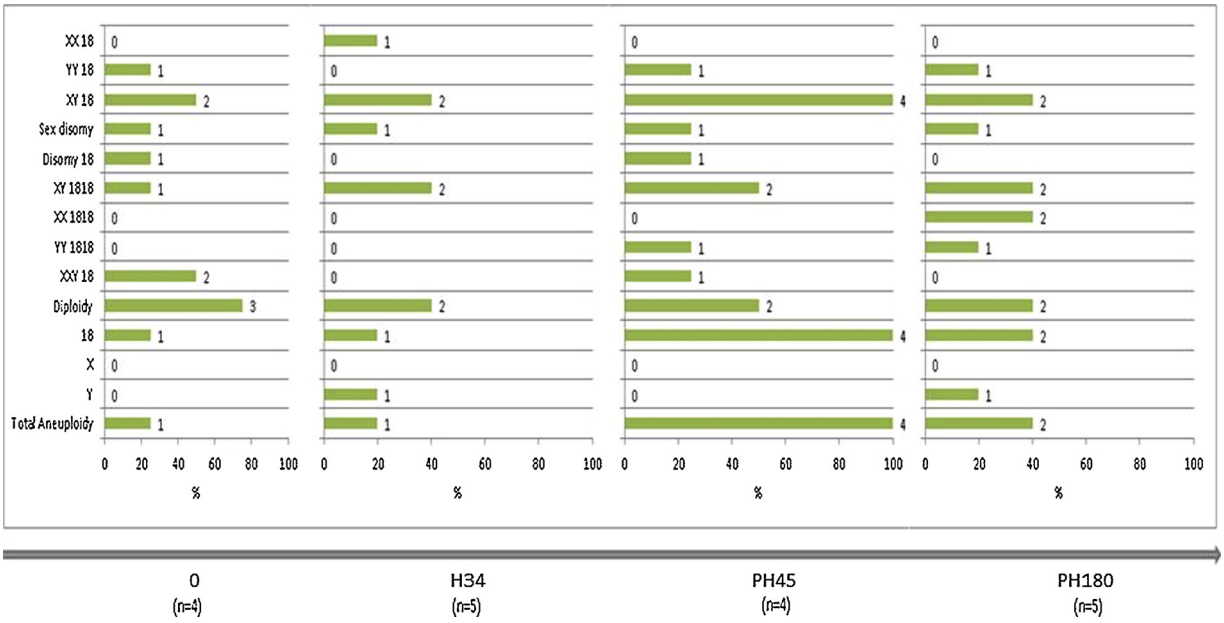

Fig. 3. Number of volunteers whose percentage of sperm aneuploidies before, during and after a mild induced increase in testicular and epididymal temperature was greater than the 90e percentile of the control group.

controls respectively.

Relation of lifestyle factors, such clothing, with sperm aneuploidy has also been reported. Jurewicz et al. [53] reported that use of boxer short decreases sperm aneuploidy rate compared to tight underwear.

In animal models, only three studies have reported the effect of induced increased temperatures on germ cell chromosomal abnormalities and abnormal chromosome configurations [42]. Exposing mice to an environment of approximately 35 ± 1 °C and 65% relative humidity for 2–5 days led to significant peaks of X-Y dissociation 5 days after the end of heat stress which recovered to control levels 50–70 days after cessation of heat stress (≈1.5–2 cycles of spermatogenesis in mice).

In another study [54], testes of mice and rats under anesthesia were heated in a water bath at 42 °C for 20 min which resulted in an increase in the percentage of X-Y univalents after 6 days of heat exposure in mice and 12 days in rats. These results suggest an action of heating in premeiotic or early prophase I stages of spermatogenesis, which was also suggested by Garriott and Chrisman [55].

The precise mechanism of aneuploidy induction in man following mild testis temperature increase is not known. However, meiosis is a structural dynamic process, broadly conserved across eukaryotes, and temperature appears to be a major challenge for chromosome segregation during the meiosis stage. Temperature increase was associated with failure of synapsis and reduced chiasma frequency in plant and animals at both high and low temperatures [56]. Heat shock proteins (HSPs) play a protective role in response to cell stress and several HSPs were induced mainly in spermatocytes and round spermatids when testis temperature was increased [57]. The HSP70 gene is expressed during the meiosis prophase, and HSP70 protein is present in the synaptonemal complex and in attachment plaques at the ends of chromosomes. Desynapsis is disrupted in male mice lacking this protein [58,59]. Moreover, heat stress following experimental cryptorchidism in rats resulted in modifications in the expression of 594 genes in pachytene spermatocytes [60].

Some cases of varicoceles are associated with abnormally elevated testes temperature. It is noteworthy that in patients with varicocele, the frequency of meiotic abnormalities such as partial asynapsis, chromosome breaks and a high rate of sex vesicle-autosome association were higher than in a control group [59]. Further analysis after varicocele surgery shoed now abnormalities suggesting that the meiotic alterations were due to increased temperature and were reversible. Likewise, a comparison of sperm aneuploidy frequency before and after varicocelectomy showed decrease, though not significant, in the sperm aneuploidy frequency [61].

One of the limitations of our study is the small number of volunteers included in this experimental protocol. The other limitation is the small number of chromosomes studied by FISH (X, Y, 18). However, the interchromosomal effect has been reported using CGH array [62,63] and it has been suggested that the results obtained by studying one set of chromosomes could be extended to others [64]. The absence of longitudinal follow-up of the control group could be another limitation of our study. Testicular temperature was not measured in this study owing to invasive approach and was based on the existing literature [30,48].

While further studies are needed to clarify the relationship between sperm aneuploidy and various circumstances resulting in testes temperature increase, findings of current study may have clinical implications. In infertile men, it seems reasonable to avoid or treat situations (fever, varicocele, ascending testis or mispositioning of testis, and occupational or professional exposure) which can result in increased scrotal or testicular temperature. If such circumstances are unavoidable, FISH analysis of sperm may be advised to identify aneuploidy which can pose possible risk to the future embryo.

In conclusion, this study shows that a mild induced increase (≈2 °C) in testicular and epididymal temperature (15 ± 1 h daily for 120 consecutive days) results in increased sperm aneuploidy in men, which reflects an effect of temperature on the meiosis stage of spermatogenesis. This effect was reversible and suggests waiting period of minimum 2 cycles of spermatogenesis if a couple is planning to conceive allowing cells sufficient time to recover from heat stress. Nevertheless, the current results are based on a smaller population which does warrant validation of these findings on larger cohort of men.

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