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Lifestyle influences human sperm functional quality

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ABSTRACT

Objective: To investigate the impact of acute lifestyle changes on human sperm functional quality. **Methods:** In the academic festivities week, young and apparently healthy male students who voluntarily submit themselves to acute lifestyle alterations (among the potentially important variations are increase in alcohol, caffeine, and tobacco consumption and circadian rhythm shifts) were used as a model system. Sperm samples were obtained before and after the academic week and compared by traditional semen analysis (n=54) and also tested for cleaved Poly ADP-ribose polymerase (PARP) protein, an apoptotic marker (n=35). **Results:** Acute lifestyle changes that occurred during the academic week festivities (the study model) resulted both in a significant reduction in sperm quality, assessed by basic semen analysis (decrease in sperm concentration, total number of spermatozoa, progressive and non-progressive motility and increase in sperm morphological abnormalities) and by an increase in the expression of the apoptotic marker, cleaved PARP, in the ejaculate. **Conclusions:** Acute lifestyle changes have clear deleterious effects on sperm quality. We propose cleaved PARP as a novel molecular marker, valuable for assessing sperm quality in parallel with the basic semen analysis method.

1. Introduction

Infertility is a complex and growing problem affecting approximately 15% of couples attempting to conceive[1]. In half of those cases, the cause is related to male reproductive issues[2]. In Portugal, it is estimated that infertility affects roughly 13%-18% of couples in reproductive age representing approximately 290 000 couples[1,2]. The high incidence of low sperm count in young European men and the declining sperm counts reported in recent decades suggest that environment in tandem with lifestyle, impact upon reproduction making this a health relevant issue[3]. In fact, lifestyle factors such as alcohol, smoking, caffeine, diet, illicit drugs, and circadian rhythm shifts induce adverse effects on male fertility[4-6]. However, the relevance of those risk factors to male fertility is still unclear.

Alcohol abuse in men has been reported to cause impaired testosterone production, oxidative stress, testes atrophy and reduction of sperm quality, resulting in infertility, impotence and reduced male secondary sexual characteristics[7,8]. Moreover, many studies have reported an association between smoking and the use of recreational drugs with testicular toxicity, sperm abnormalities and hypothalamic-pituitary dysfunction[9-15]. Yet, several inconsistent results in published data are inconclusive in correlating the effect of these factors on sperm characteristics[16-18].

Studies conducted on the general population are rare, mainly due to difficulties in sample collection and patient follow-up. In addition, for the majority of the studies, the subjects are infertile male or individuals that attend medical care, which could induce confounding factors. Finally, experiments across the different studies were not consistent with respect to the subjects tested. Some included only infertile patients, while others included

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both infertile and fertile men, precluding any meaningful comparisons between the different data sets[18–20].

In Portugal, there is a strong tradition of acute abuse of alcohol, tobacco and drug during academic festivities week. Moreover, the alcohol consumption among young people above 15 years of age in Portugal is one of the highest in Europe, representing 12.2 L of pure alcohol per person per year[21]. This offers exceptional conditions for studying this problem, since a large number of young male students voluntarily expose themselves to high quantities of harmful factors during a very well defined and limited period of time, having had a relatively healthy lifestyle before and after the exposure. Our study was aimed at determining semen ind of this self-exposed population in two moments: before (time point 1, TP_1) and after (time point, TP_2) the acute exposure scenario. The analysis of standard semen parameters such as sperm motility, concentration and morphology was used to evaluate the semen quality of individuals. However, since the evaluation of conventional seminal parameters might not be sensitive enough to establish an association between sperm quality and the abuses, we also investigated the levels of the cleaved Poly ADP-ribose polymerase (PARP) protein in human ejaculate, using it as an apoptotic marker[22–24], given that it has been previously associated with ROS (reactive oxygen species) production induced by ethanol consumption. This is also likely to contribute to a further understanding of the physiopathologic pathways that lead to the semen quality degradation, reported in this and previous studies on acute exposure to putative adverse factors[18,25].

2. Materials and methods

2.1. Study overview

The effects of several risk factors (alcohol, smoking, caffeine, diet, and circadian rhythm disturbances) on functional semen parameters (concentration, motility, and morphology) and sperm cell apoptosis were studied in samples from young male volunteers, in reproductive age, during two well defined distinct moments: before (time point TP_1) and after (TP_2) the academic festivities week held in May 2010, at the Universities of Aveiro and Coimbra, located in two of the major cities in the Centre Region of Portugal. Figure 1 summarizes the study design.

2.2. Participants

The study was advertised on the respective Campuses of the University of Aveiro and Coimbra in Portugal. Only students from those universities were accepted as volunteers participating in the study. The volunteers for this study were healthy white males, with ages ranging from 18 to 32 years. Fifty-four volunteers participated in TP_1 and TP_2. All volunteers answered an assisted fulfilment questionnaire each time a sample was provided (Figure S1 and S2; Supplementary data).

2.3. Sample collection

All participants received clear written instructions concerning the collection and transport of the semen samples and authorized the use of the samples for this study (Figure S1 and S2; Supplementary data). All volunteers donated a semen sample at each of the two time points identified above. The samples were obtained by masturbation, ejaculated into a sterile plastic container provided and maintained at room temperature until delivery to the collection points (Signal Transduction Laboratory, Aveiro or Ferticentro, Coimbra). Basic semen analysis was performed within 1 h after collection.

2.4. Questionnaires

Volunteers were asked to fill in a questionnaire (Figure S1 and S2, Supplementary data) for each time point of the study (TP_1 and TP_2). The questionnaires contained relevant past clinical information regarding periods of sexual abstinence and alcohol, tobacco and drug (both licit and

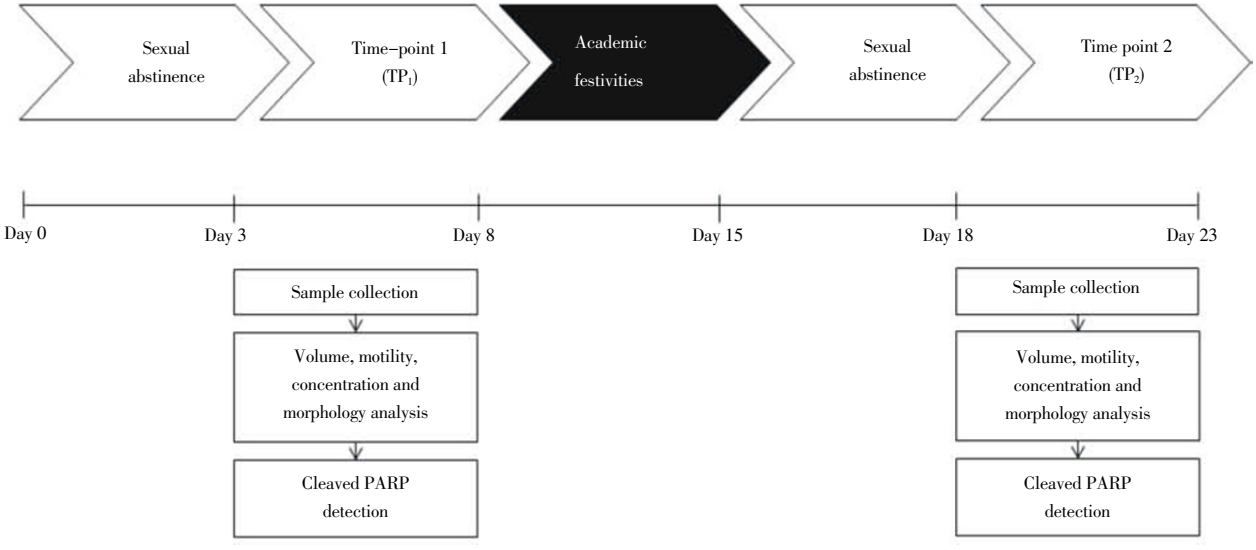


Figure 1. Diagram of the study design.

The academic week is represented by a black arrow and the other study moments by white arrows. The description of each moment is written in squares. The days of the study are indicated along the line.

illicit) consumption. The alcohol consumption parameter was defined as an estimated total amount of alcohol (in grams). In TP_1, this parameter considered the alcohol consumed per week during the month prior to the academic festivities. TP_2 reflected the alcohol consumed during the academic festivities week.

2.5. Sperm analysis

Semen analyses were performed according to the WHO (World Health Organization)'s laboratory manual for the examination and processing of human semen[26]. In order to assure comparability between determinations, all semen samples were processed and analysed by well-trained technicians whose results were consistent and comparable. To achieve complete liquefaction, the sample container was placed in a 37 °C incubator for approximately 30 min. Then, an initial macroscopic examination was performed, where the liquefaction, viscosity, appearance and volume of the semen were assessed. Further, sperm motility was evaluated by classification into three groups of motility, as recommended by WHO[26]. This parameter was measured by performing a wet preparation with an aliquot of sperm cells onto a slide, and the motility was determined by scoring the number of spermatozoa that were progressive, non-progressive (or in situ) and immotile. Concentration of sperm cells was determined by counting them using an improved Neubauer chamber, after appropriate dilution. The concentration was expressed as number of sperm cells/mL. Sperm cell morphology was evaluated, also as described by WHO. Briefly, a semen smear was applied onto a glass slide, fixed with methanol and then stained using a microscopy hemacolor kit (Merck, Germany). The incidence of normal spermatozoa and those with abnormalities in the head, neck/mid-piece and tail, were evaluated and classified.

2.6. Detection of cleaved PARP by Western blotting

Semen samples were layered over 40%/80% stepwise Percoll (Sigma) gradients[27], buffered with non-capacitating buffer, NCB (1.8 mmol/L calcium chloride, 5.4 mmol/L potassium chloride, 0.8 mmol/L Magnesium sulfate heptahydrate, 116.4 mmol/L sodium chloride, 1.0 mmol/L monopotassium phosphate, 5.6 mmol/L D(+)glucose, 2.7 mmol/L sodium pyruvate and 41.8 mmol/L sodium lactate) and centrifuged at 500 r/min for 20 min at room temperature. The supernatants were discarded and the motile sperm population was selected. The sperm pellets were washed twice in 1 mL NCB. The final pellet was resuspended in 150 μL of 10 g/L SDS and sonicated during 15 s. Protein content was determined by the BCA (Pierce) assay[28] and 50 μg were loaded on 10% SDS-PAGE. Then, samples were transferred onto nitrocellulose membranes. The latter were processed using well-established procedures and incubated with rabbit anti-cleaved PARP polyclonal antibody (Millipore, USA). Immunoreactive bands were developed by incubating with peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, UK). Beta-tubulin was used as the loading control, and detected using mouse-anti beta-tubulin monoclonal antibody (Invitrogen, USA). Due to technical constrains (insufficient protein to load on the SDS-PAGE), only 35 samples were analysed for cleaved PARP. Bands were quantified using the Quantity One densitometry software (Bio-Rad).

2.7. Statistical analysis

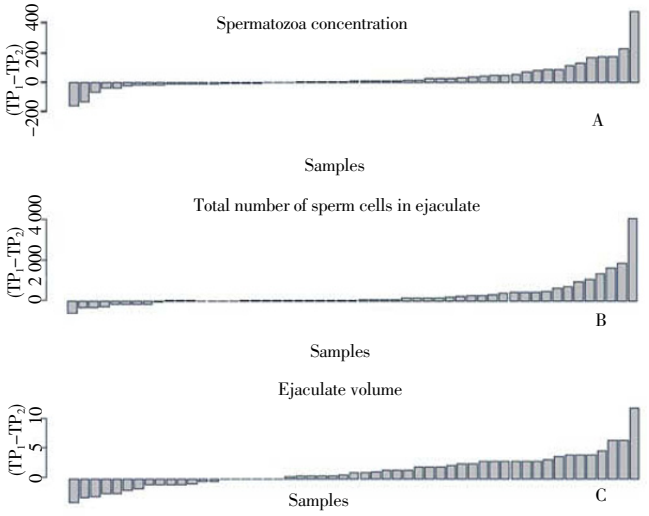
Data analysis were performed in the R language environment [29]. In order to assess the influence of the acute exposure each subject was followed (since this is a follow-up study the control-like samples are TP_1) between the two periods (TP_1-before and TP_2-after academic festivities week). Paired *t*-tests were used to assess which parameters changed between TP_1 and TP_2 for each volunteer. The use of parametric or non-parametric paired *t*-tests was judged by Shapiro-Wilk test for normality.

3. Results

3.1. Reduced sperm quality is associated with lifestyle

A total of 54 male students, with the mean age (mean± SD) of (21.7±3.0) years, participated in the study (Figure S3; Supplementary data). Differences in functional sperm parameters were observed between TP_1 and TP_2. Sperm concentration, total number of spermatozoa and volume of the ejaculate were significantly lower after the academic festivities (TP_2) compared to TP_1, with a statistical significance of P=0.008, P=0.0004 and P=0.001 2, respectively. Figure 2 depicts the sorted difference between TP_1 and TP_2 as a function of each paired sample for the three measured parameters. In all the plots, a positive bar means that the TP_1 value was higher than the TP_2 value. For the progressive motility (P=0.053) and non-progressive motility (P=0.07), a small decrease was observed from TP_1 to TP_2, although there was no significant alteration for immotility (P=0.12). The sorted differences between both time points as a function of the samples are shown in Figure 3, where it can be seen that the observed differences are balanced. In contrast, an increase in the number of sperm cells with morphological abnormalities was observed from TP_1 to TP_2, with a significant increase in spermatozoa neck/mid-piece defects (P=0.000 5), tail defects (P=0.028) and abnormal morphology (P=0.001 8). No significant differences were detected for normal and head morphological parameters (Figure 4). Finally, abstinence was found to have no significant impact between the two considered periods (P= 0.35) (Table 1).

In the present study, the extent of alcohol consumption, cigarette smoking and other conditions to which the volunteers were exposed during the festivities were assessed. The association between those factors such as alcohol consumption and smoking frequency during the academic week was further analysed. A significant 8-fold increase of alcohol amount (P=0.000 37) and a nearly 2-fold increase in smoking (P=0.001 3) was observed between TP_1 and TP_2. The results suggested the occurrence of a perturbation induced by several factors, mainly related to acute alcohol exposure and to a lesser extent, smoking, which occurred during a very restricted period of time. Although it was impossible to separate these two factors from confounding ones (*e.g.*, illicit drugs consumed, caffeine, poor diet and circadian rhythm disturbances).



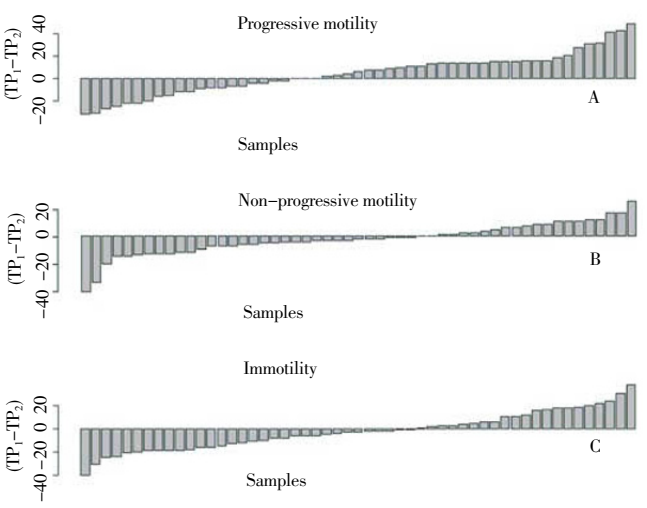
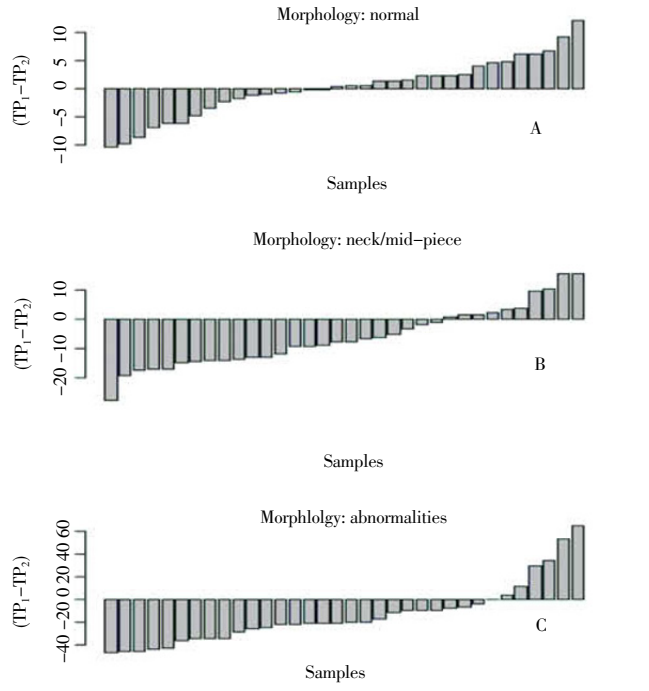


Figure 2. Bar plot of sorted differences between TP_1 (before academic week festivities) and TP_2 (after academic week festivities) as a function of samples (volunteer) per parameter evaluated.
A–spermatozoa concentration ($\times 10^6$ sperm cell/mL); B–total number of sperm cells in the ejaculated (n° sperm cells); C–ejaculate volume (mL). Each bar in the graphs represents the difference between TP_1 and TP_2 as a function of the parameter evaluated for each of the 54 volunteers.

Figure 3. Bar plot of sorted differences between TP_1 (before academic week festivities) and TP_2 (after academic week festivities) as a function of samples (volunteer) per sperm motility parameter evaluated.
A–progressive motility (n° sperm cell); B–non–progressive motility (n° sperm cells); C–immotility (n° sperm cell). Each bar in the graphs represents the difference between TP_1 and TP_2 as a function of the parameter evaluated for each of the 54 volunteers.



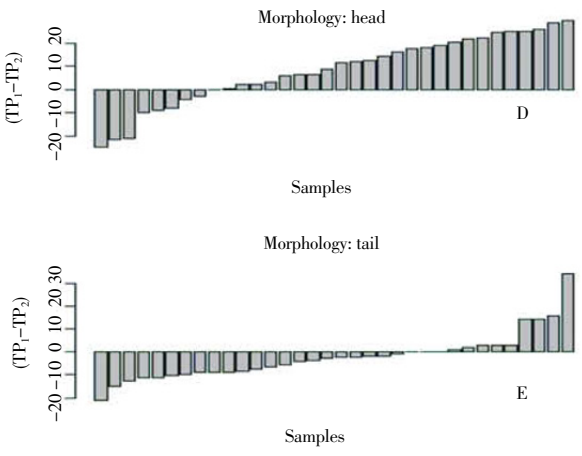


Figure 4. Bar plot of sorted differences between TP_1 (before academic week festivities) and TP_2 (after academic week festivities) as a function of samples (volunteer) per sperm morphology parameter evaluated.
A–normal morphology (n° sperm cell); B–neck/mid piece defects (n° sperm cells); C–total abnormalities (n° sperm cells); D–head defects (n° sperm cells); E–tail defects (n° sperm cells).

Table 1
Descriptive statistics for the male population in this study (n=54).

Descriptive word	Mean±SEM		Median		Variable range	
	TP_1	TP_2	TP_1	TP_2	TP_1	TP_2
Age (years)	21.6±3.0		21.0		18–32	
Sexual abstinence (d)	3.6±5.6	3.2±3.5	2.0	2.0	0–36	0–17
Volume ejaculated (mL)	4.7±2.8	3.5±2.3*	4.0	3.0	0.5–15.0	0.5–10.0

*symbolizes statistically significant difference between groups (TP_1 and TP_2) (P<0.05).

3.2. Increased cleaved PARP expression–a sperm apoptotic marker

The effect of alcohol consumption and other abusive behaviours on spermatozoa integrity was further assessed by determining the expression of cleaved PARP as an oxidative stress and apoptotic marker. The typical Western blot signal results of this study are summarized in Figures 5a and 5b. Differences are evident with respect to the amount of cleaved PARP expressed in the motile fraction of sperm ejaculated. Significant 2.4–fold (P=0.03) increase in the amount of cleaved PARP was observed in sperm ejaculated from TP_1 to TP_2. The ratio of cleaved PARP to tubulin was considered for statistical analysis purposes. The results suggested an association between alcohol consumption and other abusive behaviours during academic festivities week and increased cleaved PARP levels in ejaculated spermatozoa, a concomitant decrease in sperm concentration and an increase in defective spermatozoa.

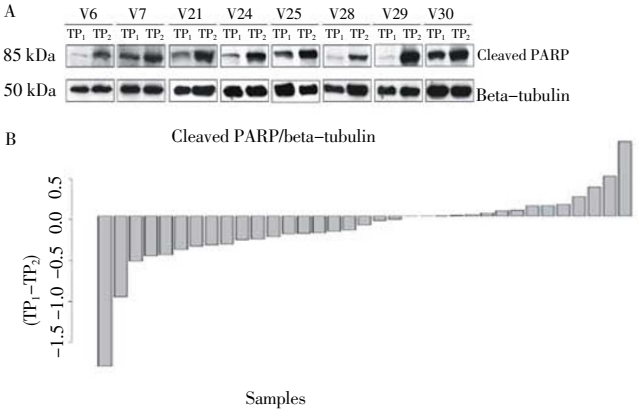


Figure 5. Cleaved PARP in human semen ejaculated (representative immunoblots).
All individual results are shown in supplementary material (Figure S4). A: volunteers, before (TP_1) and after (TP_2) academic week festivities. Immunoblot analysis was performed using anti-PARP antibody. Beta-tubulin was used as control. B: Bar plot of sorted differences between TP_1 and TP_2 of the ratio cleaved PARP and beta-tubulin, as a function of samples (volunteer, V). A significant increase (P=0.03) in the amount of cleaved PARP was observed in the samples (n=35).

4. Discussion

Excessive alcohol consumption, smoking and other abusive behaviours to which volunteers were exposed during academic festivities week had potential deleterious effects on sperm quality, leading to a statistically significant decrease in sperm concentration, total number of sperm cells in the ejaculate, volume of the ejaculate and a small decrease in progressive and non-progressive motility from TP_1 to TP_2. In addition, there was also a statistically significant increase in morphological abnormalities in the neck/mid-piece and tail. However, the number of normal sperm cells and head abnormalities were not significantly different, probably due to the very low number of normal sperm count and to the very high amount of head defects which preclude a full assessment of sperm alterations.

These results are in agreement with previous studies, where alcohol abuse was considered one of the main problems associated with poor semen production[16,17]. Moreover, the reduction in total semen volume from TP_1 to TP_2 may reflect impairment of the secretory activity of the accessory glands, namely, seminal vesicles and prostate[26]. Effectively, alcohol consumption has been shown to increase ROS, while simultaneously decreasing antioxidants, especially when associated with low nutrient diets[30], which normally occurs during the academic week. Increased ROS generation causes destructive effects on various cellular organelles. The spermatozoa plasma membrane is highly sensitive to ROS due to its high content on polyunsaturated fatty acids, susceptible to oxidative damage due to the presence of double-bounds; additionally, sperm cells lack a normal cytoplasm to generate a robust preventive and repair mechanism. ROS can damage spermatozoa biomolecules contributing to the increase in abnormal forms and induce sperm apoptosis, explaining the reduction in sperm concentration from TP_1 to TP_2[31,32]. This mechanism can be elucidated by the disruption of the mitochondrial membrane integrity, which in turn releases cytochrome c, initiating a cascade of signalling events, such as caspase 3 and 9 activation[33]. Sperm cells are more resistant to DNA damage induced by ROS compared to testicular germ cells[34]. Nevertheless, despite the tightly packed DNA and the epididymis antioxidant protection mechanisms, sperm also undergoes DNA damage. PARP, a DNA repair enzyme, have only recently been shown in ejaculated sperm samples by Mahfouz and co-workers, who demonstrated the presence of the cleaved form of PARP[24]. Our results clearly show a statistically significant increase of cleaved PARP levels in the motile fraction sperm ejaculated from volunteers from TP_1 to TP_2. Considering how sperm cells were prepared (Percoll gradient), cleaved PARP was determined in apparently "normal" sperm cells (motile), thus apoptosis is initiated already in those cells, raising interesting questions when choosing spermatozoa for assisted

reproductive techniques. PARP is activated whenever strand breaks occur in sperm DNA caused by oxidative stress, chromatin remodelling or cell death. The cleavage of PARP by caspase 3 inactivates it and inhibits PARP's DNA-repairing capacities, leading to the initiation of apoptosis. In human spermatozoa, caspase 1, 3 and 8 are localized in the post-acrosomal region[35,36]. PARP cleavage may occur by activated caspase 3 that is actively transported to the nucleus through the nuclear pores[37]. Furthermore, during the final steps of spermatogenesis, interference with PARP activation causes poor chromatin integrity with abnormal retention of histones in mature sperm[24,38]. In addition, the suppression of follicle-stimulating hormone and luteinizing hormone as well as testosterone, as a consequence of alcohol consumption, is also related to poor protamination of spermatozoa[39].

Our findings suggested a relationship between traditional semen analysis procedures and detection of cleaved PARP in human sperm. Testicular function may ultimately be evaluated through ejaculated sperm assessment. In our study, we focused on post-testicular damage, during sperm transport through the seminiferous tubules and the epididymis, as the epididymis transit takes approximately 7-10 d and time between TP_1 and TP_2 ranged from 4 to 17 d (the median and the mode were 14 d). For a better understanding of these mechanisms, studies are being carried out in order to evaluate the relationship between acute lifestyle alterations (including alcohol intake), cleaved PARP and sperm chromatin abnormalities. Thus, increased sample size studies involving subjects exposed to alcohol, smoking, drugs and circadian rhythm disturbances and diet are needed to provide a better understanding between the role of these lifestyle factors and male fertility.

The effects of lifestyle perturbations during the academic week were assessed into two different time points, TP_1 and TP_2, allowing for the toxicological effects on spermatozoa epidydimal transit and not on spermatogenesis to be addressed. The only parameter that could be quantitatively measured (by the questionnaires) was the amount of alcohol ingested by the volunteers, that rose by 8 times, and smoking that almost doubled.

The present study clearly showed that acute lifestyle changes influenced the apoptotic signalling pathways in human sperm, as evaluated by increases in cleaved PARP levels, an apoptotic marker in the ejaculated spermatozoa. Concomitantly, the same acute lifestyle changes also led to a decrease in sperm volume, concentration, total number of spermatozoa, in the progressive and non-progressive motility and in the number of normal sperm cells. Hence, these results support cleaved PARP as a molecular marker for assessing sperm quality and thus sperm function, used in parallel with traditional semen analysis. Further studies are being conducted in order to introduce cleaved PARP as a novel routine sperm quality indicator and an important tool in the clinical diagnostics of infertile couples. Hence, we propose the use of cleaved PARP as a molecular marker of sperm integrity which can be objectively measured and quantified.

Conflict of interest statement

We declare that we have no conflict of interest.

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