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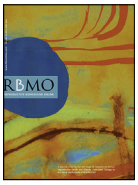

ARTICLE

Use of antioxidant could ameliorate the negative impact of etoposide on human sperm DNA during chemotherapy

BIOGRAPHY

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KEY MESSAGE

N-Acetylcysteine (NAC) was able to curb undesirable etoposide-induced in-vitro damage in human sperm chromatin and DNA. NAC may be useful as an adjuvant agent in preserving male fertility during chemotherapy treatment.

ABSTRACT

Research question: A previous study showed that *N*-acetylcysteine (NAC), used after in-vitro exposure to the gonadotoxic chemotherapeutic drug etoposide, has the ability to decrease DNA damage in human spermatozoa; however, it showed no benefit when used before exposure. This study aimed to evaluate the impact of the NAC on the preservation of sperm quality during in-vitro exposure to etoposide.

Design: Twenty semen samples were submitted to four experimental conditions: control, NAC-only incubation, etoposide-only incubation, and concomitant etoposide and NAC incubation. After in-vitro incubation, semen parameters, sperm chromatin condensation, sperm DNA fragmentation, sperm oxidative stress and sperm metabolism were used to evaluate the role of NAC in protecting human spermatozoa from etoposide.

Results: Etoposide did not affect semen parameters, nor did it cause sperm oxidative damage or alterations in glycolytic profile. However, it induced chromatin decondensation and DNA fragmentation, which were fully prevented by NAC.

Conclusions: NAC was able to protect sperm DNA integrity during etoposide treatment *in vitro*, suggesting that NAC may be useful as an adjuvant agent in preserving male fertility during chemotherapy treatments.

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KEYWORDS

Etoposide
Human sperm DNA fragmentation
Human sperm metabolism
Human sperm oxidative stress
N-Acetylcysteine

INTRODUCTION

Cancer patients often face secondary health issues emerging from cancer and its treatment, such as cancer-related infertility, that can impact their quality of life after cancer (*Anazodo et al., 2018*). Since its accidental discovery in the 1970s, cisplatin-based chemotherapy has been the first-line standard of care and has improved the prognosis of haematological (*Velasquez et al., 1994*) or solid (*Arriagada et al., 2004*; *Feldman et al., 2008*) tumours, such as testicular germ cell tumours (TGCT). First-line standards of care in cisplatin-based chemotherapy, with some refinements, are BEP (bleomycin, etoposide and cisplatin), EP (etoposide and cisplatin) and VIP (etoposide, ifosfamide and cisplatin) (*Lavoie and Kollmannsberger, 2019*). Clinical observations tend to suggest that substantial long-term and severe toxicities from chemotherapy are principally attributable to the cumulative dose of platinum-based drugs, namely cisplatin (*Chovanec et al., 2017*). This is mainly due to the persistence of reactive circulating cisplatin in numerous tissues for several years after treatment (*Travis et al., 2010*). The incidence of TGCT varies greatly with geographical location, being more prevalent in northern European countries, and is steadily increasing in young men (*Adra and Einhorn, 2017*). Successful prognosis in TGCT is mainly due to the excellent response of these solid tumours to cisplatin-based chemotherapy, with BEP or VIP being the first-line standards of care for advanced TGCT. Although TGCT are a success story in cancer treatment, with most patients cured even in highly advanced cases, cisplatin-based therapy may hinder spermatogenesis and cause lasting infertility symptoms (*Boekelheide, 2005*). However, it is relevant to mention that testicular cancer also has a deleterious impact on semen quality (*Auger et al., 2016*; *Caponecchia et al., 2016*).

Although the mechanisms of cisplatin sensitivity and resistance in tumours (*Singh et al., 2019*) and the risk of pulmonary bleomycin toxicity (*Maruyama et al., 2018*) remain important topics in the literature, further evidence is needed to clarify the role of etoposide, to determine the role of any interaction between these cytotoxic drugs that have differing mechanisms of action and to develop new strategies to reverse the side effects of chemotherapeutic agents. Etoposide is a semi-synthetic podophyllotoxin-derived agent (*Slevin, 1991*), is mitotic phase specific and was the first antineoplastic drug to be discovered whose mechanism of action is topoisomerase-II inhibition (*Martinsson et al., 2002*). Topoisomerase-II is an endonuclease that aids in DNA unwinding, being responsible for relaxing supercoiled DNA. Etoposide prevents the re-ligation of DNA strands, forming a stable complex between enzyme and DNA, which directly results in DNA strand breakage, cell cycle blockage and cell death (*Attia et al., 2012*). Etoposide can also cause DNA damage by inducing oxidative stress (*Slevin, 1991*).

Etoposide is widely used, mainly in combination, for the treatment of various neoplasms affecting males of reproductive age (*Harel et al., 2011*), such as TGCT, which, according to the 2018 World Health Organization (WHO) Global Cancer Observatory, are estimated to account for the second highest number of cancers worldwide among men under 34 years of age (*Bray et al. 2018*). Etoposide itself is known to induce male infertility (*Okada et al., 2009*) as it causes oligozoospermia and azoospermia (*Ishikawa et al., 2004*; *Stephenson et al., 1995*) and has been associated with sperm aneuploidy (*Rives et al., 2017*). In-vitro studies on etoposide are scarce, revealing only that its interaction with topoisomerase-II is responsible for human sperm distress (*Har-Vardi et al., 2007*).

Although chemotherapeutic regimens are constantly being improved, most are still harmful to male fertility (*Magelssen et al., 2006*) and, unfortunately, many men are advised to cryopreserve spermatozoa for future fertility only after the onset or after the end of the first chemotherapy regimen. The increasing incidence of cancer in reproductive age, with an increasing survival rate of patients undergoing antineoplastic therapy, points to the urgent need to preserve fertility before treatment. The germinal epithelium of the testes is highly susceptible to the deleterious effects of chemotherapy. Following administration of gonadotoxic chemotherapy, the patient may develop oligozoospermia, or even azoospermia. It is thus of the utmost importance that the patient's future with respect to his reproductive capacity be adequately addressed in the context of the complex approach to cancer management.

Addition of cytoprotectors to these therapeutic regimens is being used to preserve male reproductive capacity. *N*-Acetylcysteine (NAC) is an acetylated cysteine and a precursor of reduced glutathione (GSH) (*McClure et al., 2014*), and experimental studies have reported that NAC exhibits chemopreventive features and antioxidant activity (*De Vries and De Flora, 1993*; *Erkkilä et al., 1998*). Pharmacological studies of NAC have demonstrated that this agent is safe for clinical use (*Bonanomi and Gazzaniga et al., 1980*; *Johnston et al., 1983*), being listed on the WHO list of essential medicines. Furthermore, NAC has been shown to improve human semen parameters and protect cells from oxidative stress damage (*Ciftci et al., 2009*).

Although in-vitro toxicological studies do not allow any inference to be drawn about the impact of drugs on spermatogenesis, they are still a useful method for analysing individual drug effects, with results taken as additional information in the clinical setting. In line with this, the current group recently evaluated the in-vitro effects of etoposide on human spermatozoa and the prophylactic (NAC added before etoposide exposure) and amelioratory (NAC added after etoposide exposure) roles of NAC on the etoposide side effect profile in human spermatozoa, with results suggesting that NAC might be able to counteract etoposide-induced toxicity rather than preventing the cytotoxic effects of etoposide on sperm DNA (*Baetas et al., 2019*).

In the present report, these earlier findings have been extended to test whether concomitant use of NAC during in-vitro exposure of spermatozoa to etoposide could more efficiently protect sperm DNA. For this, semen parameters, chromatin condensation and DNA fragmentation, as well as oxidative and glycolytic profiles, were evaluated under in-vitro exposure of human spermatozoa to etoposide either alone or concomitantly with NAC.

MATERIALS AND METHODS

Ethical approval

According to Portugal's rules of the National Medically Assisted Procreation

Act (Law of 2017) and guidelines of the National Council for Medically Assisted Procreation (CNPMA-2018), clinical databases and patients' biological material for diagnosis and research may be used without further ethical approval, under strict individual anonymity and after written informed patient consent. Regarding the use of semen samples for laboratory experimentation at the Institute of Biomedical Sciences Abel Salazar-University of Porto (ICBAS-UP), the Ethics Committee gave authorization on 24 April 2019 (Project 2019/CE/P017; 266/CETI/ICBAS).

Patient selection and semen collection

Semen samples were collected by masturbation in sterile containers after a 3-day period of sexual abstinence from patients who sought semen analysis at the infertility clinic and enrolled in infertility treatments due to female factor infertility. After the semen had been liquefied, semen parameters were evaluated according to WHO guidelines (*WHO, 2010*), with detailed methodology referred to in the Checklist for Acceptability of Studies (Supplementary information and Supplementary Document 1) as recommended (*Barratt et al., 2011*; *Björndahl et al., 2016*). This was performed by experienced embryologists at the IVF clinic (Supplementary information and Supplementary Documents 2–4). For the experiments, only fresh ejaculated spermatozoa from individuals with a semen volume ≥1.5 ml, sperm concentration ≥15 × 10^6/ml and progressive motility ≥32% were used. Other male exclusion criteria were the existence of known pathologies and medication intake, anatomic abnormalities, hormone profiles and karyotypes, and the presence of semen agglutination, immature forms, leukocytes and micro-organisms (*WHO, 2010*). This selection provided a total of 20 semen samples with total normozoospermic parameters, similar to a donor population, although without proven offspring. Power analysis was used to estimate an adequate sample size using the free software G*Power version 3.1.9.4 (*Faul et al., 2007*).

Unless otherwise stated, all chemicals were purchased from Merck (Germany).

Experimental design

After semen analysis, the remaining ejaculate from each patient was centrifuged at 380*g* for 5 min to discard the seminal fluid. The resulting pellet was resuspended in 1 ml of prewarmed sperm preparation medium (SPM; Origio, Denmark) and diluted. All samples were individually submitted to the same experimental procedure after dilution to a sperm concentration of 10 × 10^6/ml to avoid any deviation from the specific concentration of etoposide or NAC. Experiments were repeated 20 times.

Each sperm sample was divided up to be exposed to four different experimental conditions. Each experiment lasted 2 h and was performed in a humidified incubator with 5% CO_2 at 37°C. The control group (CT) consisted of spermatozoa incubated with SPM; the Eto group involved incubation of spermatozoa with 25 µg/ml etoposide (Sigma, USA); the NAC group had incubation of spermatozoa with 50 µmol/l NAC (Sigma); and the Eto+NAC group involved incubation of spermatozoa with 25 µg/ml etoposide plus 50 µmol/l NAC. The etoposide concentration of 25 µg/ml used in the present in-vitro experiments was that considered pharmacologically and physiologically relevant for human therapeutic doses, and was based on peak serum etoposide concentrations, which range from 17 to 88 µg/ml (*Joel, 1966*; *Open Chemistry DataBase 2020*). The exposure time was based on toxicological studies showing that the highest bioavailability of etoposide occurred within the first 2 h, with the peak plasma concentration being attained by 1–1.5 h (*Open Chemistry DataBase 2020*; *Slevin, 1991*). For NAC, the 50 µmol/l dose was that previously determined to maximize the results without damaging the spermatozoa (*Dorato and Engelhardt, 2005*). Procedures for proper handling and disposal of the antineoplastic drug etoposide were considered during the course of the present investigation, with specific security facilities and handling being used.

In the experiments, the following characteristics were evaluated in control and treated sperm samples: sperm progressive motility, hypo-osmotic swelling test (HOST) results, chromatin condensation, sperm DNA fragmentation (sDNAfrag), and glycolytic and oxidative profiles.

Determination of sperm chromatin condensation

Sperm chromatin condensation was assessed by acidic aniline blue staining according to the procedure described by Baetas and colleagues (*Baetas et al., 2019*). At least 200 spermatozoa per slide were evaluated in a blinded manner. The percentages of sperm heads that stained dark blue (indicating immature histone-rich nuclei) and those that remained unstained (indicating protamine-rich mature chromatin) were calculated. In this test, controls were not used. Discrimination between lysine-rich histones (aniline blue positive) and arginine-rich protamines (aniline blue negative) reveals the level of maturity of the sperm chromatin, which is an important factor for vulnerability to sDNAfrag and abnormal embryo development (*Hofmann and Hilscher, 1991*; *Selami et al., 2013*; *Zidi-Jrah et al., 2016*). A single experienced researcher (R.S.) supervised these experiments.

Determination of sDNAfrag

The incidence of morphologically normal spermatozoa displaying nuclear DNA strand breaks was identified using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay as previously described (*Gomes et al., 2014*; *Sá et al., 2015*). For each experiment, negative (terminal deoxynucleotidyl transferase enzyme omitted and replaced by distilled water) and positive (deoxyribonuclease treatment) controls were performed to ensure reproducibility of the assay. On each slide, a minimum of 200 spermatozoa were scored in a double-blind manner, and each spermatozoon was assigned as either having DNA fragmentation (if displaying intense green fluorescence) or being normal (4',6-diamidino-2-phenylindole [DAPI] staining only). The percentage of TUNEL-positive spermatozoa was expressed as the percentage of cells exhibiting sDNAfrag. The TUNEL test presents low inter- and intraobserver variability, presenting consistent results in different countries. This test is considered a highly valuable indicator of sperm quality, with increased sDNAfrag being related to poor embryo development and clinical outcomes (*Majzoub et al., 2017*; *Sergerie et al., 2005*; *Zidi-Jrah et al., 2016*). A single experienced researcher (R.S.) supervised these experiments.

Definition of normal sperm morphology during the assessment of sperm chromatin condensation and sDNAfrag

Only spermatozoa with normal morphology were counted during

sDNAfrag and aniline blue assessment because, for assisted reproductive technology (ART) treatments, this group uses purified spermatozoa obtained after differential gradient centrifugation followed by swim-up. This means that only highly motile and grossly morphologically normal spermatozoa (the upper part of the swim-up fraction) are used. For ART as well as the current experiments, the term 'morphologically normal spermatozoa' was used to discriminate them from spermatozoa showing gross anomalies in the sperm head (irregular, small, enlarged, decapitated), midpiece (cytoplasmic droplets, enlarged) or tail (bent, short, enlarged, multiple). These gross anomalies are easily observed on aniline blue and DAPI staining. The TUNEL experiments used a fluorescent filter that does not capture the highest wavelength frequency of fluorescein isothiocyanate emission light but captures the low frequencies of the emission spectrum; thus, the morphology of individual spermatozoa could be evaluated without interfering with the emission spectrum or degradation of the fluorophore (*Bucar et al., 2015*; *Sá et al., 2015*).

Determination of oxidative stress markers

Oxidative stress can be assessed by calculating the cell protein carbonyl content, a marker of protein oxidation; by measuring aldehyde products, such as 4-hydroxynonenal (4-HNE), an indicator of lipid peroxidation; and by quantification of 3-nitrotyrosine, an indicator of superoxide-dependent peroxynitrite formation. The respective contents of these were evaluated using the slot-blot technique and specific antibodies, as previously reported (*Dias et al., 2015*). To evaluate the carbonyl groups, protein samples were derived using 2,4-dinitrophenylhydrazine to obtain 2,4-dinitrophenyl (DNP) according to previous descriptions by Levine and collaborators (*Levine et al., 1990*).

The slot-blot analysis was performed using a hybrid-slot manifold system (Biometra, Germany), and the resulting polyvinylidene fluoride membranes were incubated overnight at 4°C with a rabbit anti-DNP (1:5000) (Sigma). To analyse lipid peroxidation and protein nitration, protein samples were diluted in phosphate-buffered saline to a concentration of 3 µg/µl. The resulting membranes were incubated overnight at 4°C with goat anti-4-HNE antibody (1:5000) and rabbit nitrotyrosine antibody (1:5000) (Cell Signaling Technology, USA). Samples were visualized using rabbit anti-goat immunoglobulin (IgG-Alkaline Phosphatase; 1:5000) (Sigma) or goat anti-rabbit IgG-Alkaline Phosphatase (1:5000) (Santa Cruz Biotechnology, Germany). Membranes were then reacted with ECF substrate (GE Healthcare, UK) and read using a Gel Doc XR+ (Bio-Rad, UK). The densities from each band were quantified using Quantity One Software Version 4.6.9 (Bio-Rad, UK). Using this methodology, a positive control is not used. Two negative controls were performed, one without a cell sample and the other without a primary antibody. Results are presented as fold variations relative to the control group.

Sperm metabolism

After incubation, extracellular metabolites were acquired by proton nuclear magnetic resonance (^{1}H-NMR), as previously described (*Alves et al., 2013*). Sodium fumarate was used at a final concentration of 1 mmol/l as an internal reference (6.50 ppm) to quantify the following metabolites (multiplet, ppm): H1-α-glucose (doublet, 5.22), choline (singlet, 3.18), pyruvate (singlet, 2.38),; acetate (singlet, 1.9) and lactate (doublet, 1.33 ppm). The relative areas of the ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTS-Pro NMR spectral analysis program (Acorn NMR, USA).

Statistical analysis

Statistical significance among the experimental groups was assessed by analysis of variance, followed by Fisher's least significant difference. All data are shown as mean ± SEM. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Patient characteristics

The initial (clinical) evaluation of the semen parameters is detailed in TABLE 1. No significant differences were found between male ages, semen volume, sperm concentration, motility, normal morphology, vitality and HOST results. Sperm morphology was not evaluated in the experimental groups' mainly because quantifiable sperm morphological changes are not expected to occur in a short period of exposure. Additionally, changes in chromatin condensation and DNA fragmentation are unrelated to substantial morphological changes in head appearance, and etoposide does not interfere with microtubular assembly or membrane integrity.

Effects on sperm motility

No significant differences were observed in the mean percentage of progressive motile spermatozoa between the groups: controls (18.63 ± 3.81), Eto (19.60 ± 4.52), NAC (22.40 ± 5.85) and Eto+NAC (22.74 ± 5.96) (FIGURE 1A).

Effects on sperm membrane integrity

Relative to the control group (47.07 ± 2.74), no significant differences were found in the mean percentage of live spermatozoa for the Eto (52.38 ± 3.41), NAC (46.30 ± 2.68) and Eto+NAC (45.71 ± 3.81) groups. However, relative to the Eto group, the mean percentage of viable spermatozoa was significantly reduced in the NAC ($P = 0.04$) and Eto+NAC ($P = 0.03$) groups (FIGURE 1B).

Effects on sperm chromatin condensation

A significantly higher mean percentage of spermatozoa with uncondensed (immature) chromatin was observed in the Eto group (26.60 ± 2.77) compared with the control (17.20 ± 1.22; $P = 0.02$), NAC (17.47 ± 3.11; $P = 0.04$) and Eto+NAC (15.76 ± 1.94; $P < 0.0001$) groups (FIGURE 1C). No significant difference was found between the other groups (Supplementary information).

Effects on sDNAfrag

The mean percentage of sDNAfrag was significantly higher in the Eto group (15.93 ± 0.74) compared with the control (10.57 ± 2.05; $P = 0.0019$), NAC (10.31 ± 1.63; $P = 0.02$) and Eto+NAC (10.56 ± 1.63; $P = 0.02$) groups (FIGURE 1D). No significant difference was found the other groups (Supplementary information).

Effects on sperm oxidative stress

Sperm exposure to etoposide did not increase protein nitration levels compared with controls (1.04 ± 0.05-fold variation versus the control group). However, compared with the Eto group, significantly higher levels of protein nitration were observed in the NAC (1.12 ± 0.05-fold variation from control levels; $P = 0.04$) and Eto+NAC (1.19 ± 0.06-fold variation from controls; $P = 0.02$) groups (FIGURE 1E).

TABLE 1 DEMOGRAPHIC DATA AND BASIC SPERM PARAMETERS AT THE TIME OF COLLECTION

Variable	Mean	SEM	*P*-value*
Age (years)	33.8	1.2	0.922
Volume (ml)	3.8	0.3	0.338
No. of spermatozoa/ml	92.2	21.5	0.064
Motility (%)			
Immotile	31.1	2.9	0.137
Non-progressive	22.6	2.9	0.135
Progressive	46.3	4.9	0.727
Normal morphology (%)	4.8	0.9	0.804
Teratozoospermia index (%)	1.6	0.0	0.425
Vitality (%)	74.9	1.8	0.268
Hypo-osmotic swelling test (%)	70.3	2.7	0.303

* Significance at $P < 0.05$.

No significant differences were observed between the groups regarding sperm carbonyl values for the Eto (0.91 ± 0.05-fold variation from control), NAC (0.99 ± 0.04-fold variation from control) and Eto+NAC (0.95 ± 0.04-fold variation from control) groups (FIGURE 1F).

No significant differences between groups were observed regarding sperm lipid peroxidation in the Eto (1.01 ± 0.05-fold variation from control), NAC (1.02 ± 0.04-fold variation from control) and Eto+NAC (0.99 ± 0.06-fold variation from control) groups (FIGURE 1G).

Effects on sperm metabolism

Glucose consumption was 32.02 ± 5.17 pmol/10^6 spermatozoa in the control group, 31.33 ± 8.32p mol/10^6 spermatozoa in the Eto group, 32.63 ± 4.47 pmol/10^6 spermatozoa in the NAC group and 48.33 ± 11.25 pmol/10^6 spermatozoa in the Eto+NAC group, with no significant differences between groups (FIGURE 2A).

Pyruvate consumption was 10.63 ± 1.96 pmol/10^6 spermatozoa in the control group, 11.51 ± 3.05 pmol/10^6 spermatozoa in the Eto group, 11.78 ± 2.47 pmol/10^6 spermatozoa in the NAC group and 16.54 ± 4.11 pmol/10^6 spermatozoa in the Eto+NAC group, with no significant differences between groups (FIGURE 2B).

Lactate production in the control group was 11.21 ± 1.01 pmol/10^6 spermatozoa, with no significant differences observed between the Eto (9.20 ± 1.16 pmol/10^6 spermatozoa), NAC (10.56 ± 1.31 pmol/10^6 spermatozoa) and Eto+NAC (9.60 ± 0.64 pmol/10^6 spermatozoa) groups (FIGURE 2C).

Acetate production in the control group was 0.001 ± 0.001 pmol/10^6 spermatozoa, with no significant differences observed between the Eto (0.002 ± 0.001 pmol/10^6 spermatozoa), NAC (0.002 ± 0.001 pmol/10^6 spermatozoa) and Eto+NAC (0.005 ± 0.001 pmol/10^6 spermatozoa) groups (FIGURE 2D). However, the Eto+NAC group showed a significant ($P < 0.001$) 4.0-fold increase in acetate production compared with the control group (FIGURE 2D).

Choline production in the control group was 0.44 ± 0.10 pmol/10^6 spermatozoa. Similar values were observed in the Eto group (0.43 ± 0.13 pmol/10^6 spermatozoa). The NAC group showed significantly higher choline production (1.08 ± 0.42 pmol/10^6 spermatozoa; $P = 0.04$) compared with all other groups. Although the Eto+NAC group presented the lowest values for choline production (0.12 ± 0.07 pmol/10^6 spermatozoa), this difference was only significant compared with the NAC group (FIGURE 2E).

DISCUSSION

Etoposide is used in combined therapeutic schemes to treat several neoplasms in patients of reproductive age, such as testicular tumours (*Liu and Joel, 2003*). After treatment, these patients show decreased sperm quality (*Sieniawski et al., 2008*; *Paoli et al., 2016*) and high incidence of morphologically abnormal sperm (*Stephenson et al., 1995*). Due to the combined nature of the treatments, the direct adverse effects of etoposide on human spermatozoa have remained unknown.

In an attempt to provide information on the direct effects of etoposide on human spermatozoa and to evaluate the potential benefits of adjuvant antioxidant therapy, this group published a previous report analysing the effects of sperm exposure to etoposide under two different conditions: with the antioxidant NAC administered before or after exposure to etoposide. When NAC was added after etoposide exposure, there was an absence of deleterious effects on sperm motility and membrane integrity, with a significant increase in sperm maturity and an apparent, although non-significant decrease in sDNAfrag and oxidative values (*Baetas et al., 2019*). The current study was designed to address unresolved issues and complement this initial study.

In this study, NAC was added concomitantly during sperm exposure to etoposide to test whether this approach could achieve a stronger protective effect over the spermatozoa. The present results show that under these conditions, besides not compromising sperm motility, sperm viability and oxidative and metabolic profiles, the significant increase in uncondensed chromatin and sDNAfrag induced by etoposide was totally prevented by co-incubation with NAC. The data thus suggest, as previously indicated, that the results are substantially better when NAC is used concomitantly with etoposide rather than after etoposide exposure (*Baetas et al., 2019*). These results therefore show that using NAC to counteract etoposide-induced sperm DNA damage is a promising strategy. Notwithstanding, future studies must assess the effects of NAC with other cisplatin-based therapy drugs and the clinical effects of NAC in patients enrolled on cisplatin-based therapies. However, the present results may enhance the usefulness of NAC in combination with etoposide in significantly reducing the deleterious effects of etoposide on sperm DNA, and therefore in making clinical decisions on fertility preservation in patients at risk of losing their fertility potential due to cancer treatment.

The reduction in sperm quality after chemotherapy is generally attributed to DNA damage, which causes the cells to

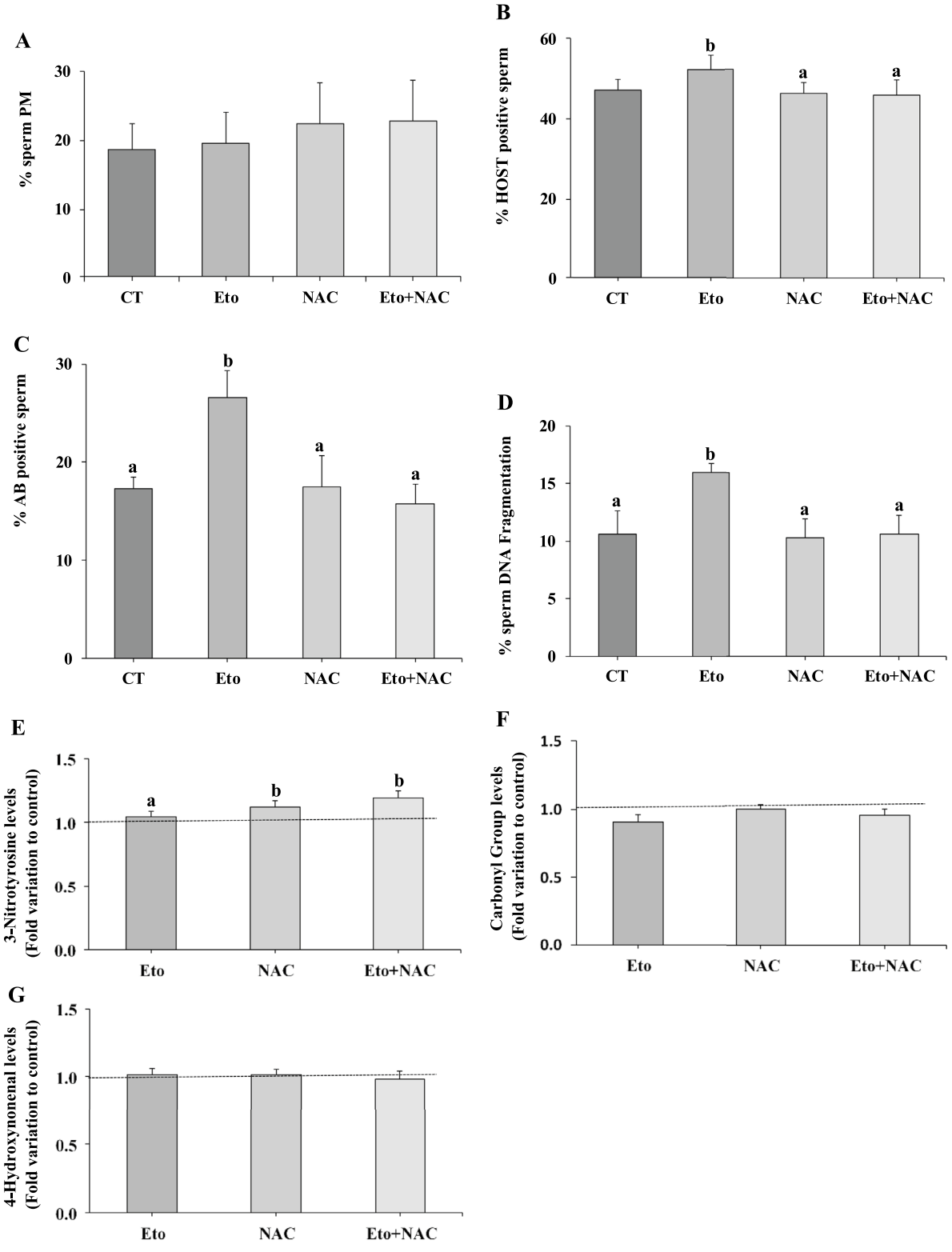

FIGURE 1 Effect of exposure to etoposide and N-acetylcysteine on human sperm parameters and DNA integrity (A–D), and on oxidative stress markers in spermatozoa (E–G), after 2 h of incubation. (A) sperm progressive motility (PM); (B) sperm membrane integrity assessed by the hypo-osmotic swelling test (HOST); (C) immature chromatin condensation in spermatozoa assessed by aniline blue (AB) staining; (D) sperm DNA fragmentation assessed by TUNEL assay; (E) protein nitration; (F) protein oxidation; (G) lipid peroxidation. CT, control group; Eto, spermatozoa treated with 25 µg/ml etoposide; NAC, spermatozoa treated with 50 µmol/l NAC; Eto+NAC, spermatozoa treated with 25 µg/ml etoposide and 50 µmol/l NAC. (E–G) Results presented as fold variation from the control group (dotted line, value 1.0). Data are displayed as mean ± SEM. Significant differences ($P < 0.05$) between experimental groups are indicated by letters over corresponding columns (a = a; b = b; a ≠ b). n = 20 semen samples.

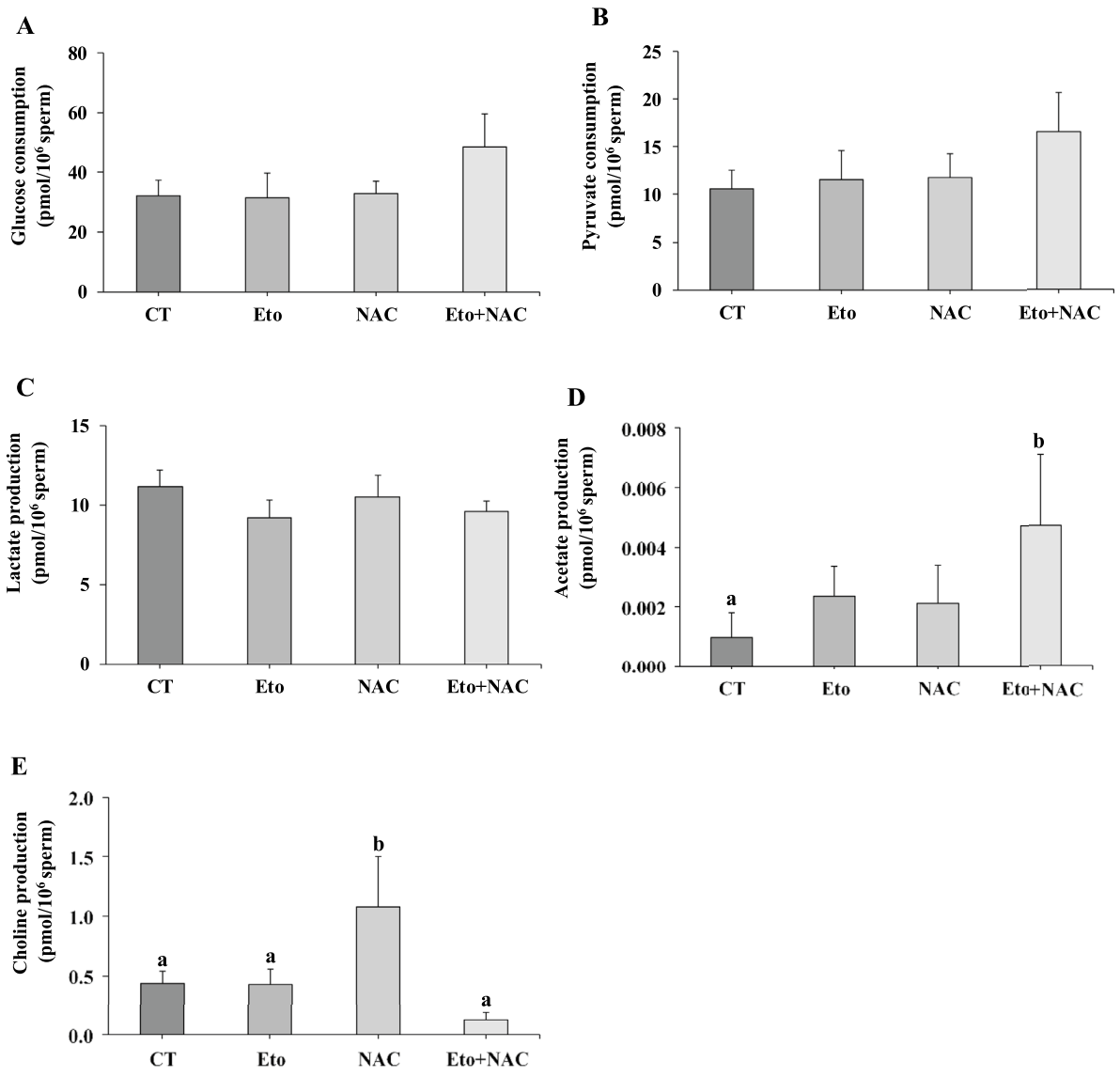

FIGURE 2 Effect of etoposide and N-acetylcysteine exposure on sperm metabolism, after 2 h of incubation. (A) glucose consumption; (B) pyruvate consumption; (C) lactate production; (D) acetate production; (E) choline production. CT, control group; Eto, spermatozoa treated with 25 µg/ml etoposide; NAC, spermatozoa treated with 50 µmol/l NAC; Eto+NAC, spermatozoa treated with 25 µg/ml etoposide and 50 µmol/l NAC. (E–G) Results presented as fold variation from the control group (dotted line, value 1.0). Data are displayed as mean ± SEM. Significant differences ($P < 0.05$) between experimental groups are indicated by letters over corresponding columns (a = a; b = b; a ≠ b). n = 20 semen samples.

undergo apoptosis, rather than necrosis (*Marchetti et al., 2006*). Studies in mice have shown that in-vitro exposure to etoposide results in spermatocyte DNA damage and chromatin modifications (*Matulis and Handel, 2006*). Moreover, the administration of therapeutic doses of etoposide *in vivo* increased DNA fragmentation in rat testicular cells (*Okada et al., 2009*), and increased spermatocyte chromosomal aberrations (*Marchetti et al., 2006*) and mutations (*Russel et al., 1998*) in mice. The current results in humans also show a significant increase in the proportion of spermatozoa with uncondensed chromatin and sDNAfrag. However, it remains to be determined whether breaks in the DNA, which may increase access to aniline blue, are enabling a false increase in chromatin decondensation or whether the increase in the percentage of spermatozoa with uncondensed chromatin is facilitating sDNAfrag.

Etoposide is believed to induce DNA damage through the formation of reactive oxygen species (ROS), with a subsequent induction of oxidative stress (*Slevin, 1991*). A study with rat thymocytes exposed *in vitro* to etoposide reported the formation of hydroxyl radicals, with induction of apoptosis (*Wolfe et al., 1994*), and another in-vitro hepatic cell study in mice reported the onset of DNA nicking (*Sinha et al., 1983*). Here, evidence is provided that human sperm exposed *in vitro* to etoposide do not show an altered oxidative profile in terms of protein carbonylation and lipid peroxidation. Therefore, the current results corroborate the suggested mechanism of preventing the re-ligation of DNA strands, resulting in DNA strand breaks, and therefore in activation of the caspase-mediated apoptosis pathway. Nonetheless, and although there were no significant differences in protein

nitration between the control and Eto groups, there was a significant increase in peroxynitrite values in the NAC and Eto+NAC groups, with no significant differences between them, suggesting that NAC is probably exerting a pro-oxidant effect. However, as NAC is able to restore sperm GSH concentrations (*Baetas et al., 2019*), it is likely that it will be possible to scavenge the cell-toxic peroxynitrite with continuous NAC treatment. Indeed, the present results cannot rule out the mechanism of oxidative stress damage, and more sensitive methods for testing oxidative stress should be considered in future work. Further studies are therefore needed to unravel this issue and to understand whether caspase-3 and caspase-9 activation and investigation of the Bax/Bcl-2 ratio will allow researchers to discern the actual mechanism behind the etoposide-induced increase in sDNAfrag, be it either an excessive DNA breakage in individual cells or an apoptotic process.

The antioxidant activity of NAC is used as a protective supplement in several diseases. Prevention of DNA damage by NAC is thought to rely on the replenishment of intracellular antioxidant mechanisms (*De Flora et al., 2001*). Studies in human sperm *in vitro* (*Lopes et al., 1998*) and after oral administration of NAC (*Ciftci et al., 2009*) have suggested that the antioxidant properties of NAC are responsible for the reduction in sDNAfrag. Similarly, other in-vitro studies on human spermatozoa have demonstrated that the addition of NAC also decreases ROS levels (*Oeda et al., 1997*), with the same results being observed in human cell lines (*Talley et al., 1995*). This study confirms the protective role of NAC, as its addition fully shielded human sperm DNA against chromatin decondensation and DNA fragmentation induced by etoposide. In this context, it is important to stress that NAC prevents overall sDNAfrag without distinguishing between single-strand DNA breaks and double-strand DNA breaks, and that it does not necessarily prevent apoptosis.

Under certain conditions, NAC may have antioxidant or pro-oxidant properties. For instance, a mouse study showed that, *in vivo*, NAC exerts a strong antioxidant defence in cells under increased nitrative stress (*Abdelmegeed et al., 2013*). Other studies have reported that, in non-oxidant milieus, NAC may have a pro-oxidant activity, generating hydrogen peroxide and increasing the secretion of nitric oxide and cellular nitrotyrosine, as has been shown in cow luteal cells (*Löhrke et al., 2010*) and rat glial neuronal cells (*Sagara et al., 2010*). This means that NAC can interfere differently with intracellular pathways in different cell types. The data from the current study suggest that, in human spermatozoa submitted to a pro-oxidant milieu (etoposide exposure), NAC may act at different cellular levels, as it did not interfere with the motility machinery, negatively impacted on membrane resilience, induced protein nitration and prevented DNA damage. As exposure to etoposide did not alter the oxidative status of the spermatozoa, the results also suggest that etoposide may directly affect chromatin condensation and DNA fragmentation in spermatozoa through mechanisms not mediated by oxidative stress.

Although NAC did not affect sperm viability in relation to controls, the mean percentage of viable spermatozoa was significantly reduced in the NAC and Eto+NAC groups in comparison to the Eto group, probably due to a pro-oxidant activity of NAC. One plausible explanation is that Eto is more lipophilic than NAC, and as its deleterious effects are on the sperm DNA rather than the sperm cell membrane, the functional integrity of the human sperm membrane is maintained; as measured by the HOST test, this gives better results than with NAC alone or with Eto+NAC. Another explanation for this pro-oxidant activity of NAC could be that the sperm membrane is more susceptible to oxidative damage because it is rich in polyunsaturated fatty acids and because spermatozoa display low concentrations of scavenging enzymes (*Linhartova et al., 2015*).

Clinically, NAC has been reported as having potential protective effects in several diseases, such as diabetes mellitus and its associated complications (*Lasram et al., 2015*), chronic obstructive pulmoray disease (*Calzetta et al., 2018*) and cystic fibrosis (*Hurst et al., 1967*). Therefore, in a cell, such as the spermatozoon, that lacks self-repair mechanisms, the ability of NAC to prevent or block the deleterious effects of etoposide exposure is strong evidence for its use in adjuvant therapies.

Sperm metabolism is essential to obtain the energy required for regular functions such as motility, with glycolysis being the preferred route (*Dias et al., 2014*). In somatic cells (B-cell lymphoma), high concentrations of glucose in culture medium have been reported to protect against the deleterious effects of etoposide (*Shao et al., 2014*). Choline metabolism is also important in spermatozoa as it regulates sperm membrane structure and fluidity, and is crucial for sperm functions (*Lazaros et al., 2012*). This has shown been shown in male mice with a knockout of the gene for choline dehydrogenase, leading to infertility (*Johnson et al., 2010*).

The current results show that glucose and pyruvate consumption, as well as lactate, acetate and choline production, was not altered after exposure to etoposide or NAC, except for choline production in the presence of NAC. These results illustrate that neither etoposide nor NAC negatively affected the glycolytic profile of human spermatozoa. To the researchers' knowledge, this is the first report of the effects of etoposide or/and NAC on glucose metabolism in spermatozoa. Although the result was not significant, the combination of NAC and etoposide was associated with a 1.5- and 1.6-fold increase in glucose and pyruvate consumption, respectively, in relation to controls, suggesting a protective effect of NAC. The significant increase in acetate production also observed in the Eto+NAC group, along with the protective effects over DNA integrity, suggests that spermatozoa depend on the metabolism of acetate to survive, as this carbon source can contribute to the production of acetyl-coenzyme A. Thus, in the presence of etoposide, NAC increases acetate production to counteract the toxic effects of etoposide.

Another curious observation was the increased production of choline caused by NAC. Phosphatidylcholine is the most abundant phospholipid in eukaryotic membranes, and is essential for the structural integrity and signalling functions of cell membranes (*Li and Vance, 2008*). This isolated action of NAC suggests that it alone seems to be inhibiting the enzyme choline phosphotransferase, and thus phosphatidylcholine biosynthesis, leading to an accumulation of its substrate, choline. This probably represents a new mechanism of action that may explain

why, when used alone or without the cells being under stress, NAC has a pro-oxidant rather than an antioxidant effect. Nevertheless, further studies are required to investigate this mechanism. Conversely, in the presence of etoposide, which would be expected to inhibit phosphatidylcholine biosynthesis directly by intracellular acidification after induction of apoptosis (*Anthony et al., 1999*) and thus lead to an accumulation of choline, NAC seems to counteract this effect, displaying an antioxidant effect in cells already under stress. This dual action is further supported by the present observation that NAC abolished etoposide-induced sDNAfrag. In this context, NAC would be protecting chromatin against etoposide-induced damage by favouring the biosynthesis of phosphatidylcholine and thus the integrity of the nuclear envelope.

In addition, NAC can act as a direct or indirect antioxidant (as it is a precursor of GSH), but especially as a agent detoxifying electrophilic molecules (such as the quinone-amine of paracetamol). Etoposide is metabolized to cytotoxic metabolites, including the corresponding quinone metabolite (*Zheng et al., 2006*), which is a covalent poison of human topoisomerase IIβ and is capable of inducing a higher ratio of double-strand breaks (*Smith et al., 2014*). Reactive quinone is metabolized by GSH as already reported (*Zheng et al., 2006*) and probably also by NAC. The previously reported suggestive biological beneficial effect of NAC on sperm DNA damage induced by etoposide (*Baetas et al., 2019*) is fully confirmed here. Although this study also intended to be a mechanistic insight into the protective effect of NAC on etoposide-induced DNA damage, this mechanism should be further considered and tested. This should, for example, evaluate the oxidative metabolism of etoposide in terms of the formation of the metabolite quinone in spermatozoa, as well as measure the NAC adduct with the metabolite quinone of etoposide, using mass spectrometry.

This group's previous study (*Baetas et al., 2019*), which used a sequential addition of etoposide and NAC (with NAC added before or after etoposide exposure), revealed that administration of NAC to cells after etoposide exposure is preferable to its prophylactic use. In the current study, the co-incubation strategy (concomitant administration) adopted does not allow an assessment of whether NAC reduces the bioavailability of etoposide, preventing its detrimental impact on DNA integrity, or whether it alleviates damage after its induction. Given the cooperative results, it can be stated that concomitant addition of NAC is especially effective as it completely abolished the adverse effects of etoposide; this can be suggested as the main basis of using NAC as adjuvant therapy. Nevertheless, it is important to note that previous results indicated that the prophylactic use of NAC is not beneficial, and that NAC still confers protection in those patients who have completed treatment (*Baetas et al., 2019*). It would also be of interest to discover the relevance of choline metabolism and NAC in human spermatozoa, and to measure cellular ATP concentrations to reveal its full significance on sperm metabolism following exposure. Likewise, future work should also explore different concentrations and exposure times. This study was not designed to evaluate possible consequences in terms of clinical outcomes. However, it would be of interest to investigate the adjuvant therapeutic effects of NAC on TGCT patients treated with cisplatin-based chemotherapy.

CONCLUSION

Etoposide is a cornerstone of fertility-impairing cisplatin-based therapies in several tumours of male reproductive age. The current study was able to confirm the isolated deleterious effect of etoposide on human sperm DNA and the usefulness of NAC in protecting human spermatozoa from this side effect of etoposide. This ability of NAC to preserve human spermatozoa from this etoposide-induced damage may be of clinical relevance (*Palo et al., 2005*; *Trottmann et al., 2007*). NAC presents great advantages as it is an inexpensive drug, is already approved for safe human use and has minimal adverse side effects. Use of NAC as adjuvant treatment during cisplatin-based chemotherapy and oncological prophylactic sperm cryopreservation could be investigated in clinical studies. Ultimately, it is possible also to suggest a clinical benefit of NAC use in infertile patients with high sDNAfrag levels, as well as in sperm preparation and cryopreservation media.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2020.01.029.

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