Original Paper

Hyperoside Supplementation in Preservation Media Surpasses Vitamin C Protection Against Oxidative Stress-Induced Damages in Human Spermatozoa

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Key Words

Assisted-reproduction • Hyperoside • Vitamin C • Sperm-quality

Abstract

Background/Aims: Oxidative Stress (OS) is reported as one of the main causes of male infertility. Infertile couples often resort to assisted reproductive technology (ART) to achieve parenthood. However, preparation for ART protocols increases the exposer of gametes to OS. Thus, it is crucial to find suitable preservation media that can counteract the OS-induced damages in spermatozoa. In this work, we tested and compared the efficiency of vitamin C (VC) and hyperoside (HYP) as potential antioxidant supplements for sperm preservation media. **Methods:** We evaluated the cytotoxicity of HYP (0, 5, 50, 100, and 500 μM) in spermatozoa. After incubation of sperm cells with VC (600 μM) and HYP (100 and 500 μM), in the presence and absence of H_2O_2 (300 μM), the following parameters were assessed: total sperm motility and vitality, OS biomarkers expression, total antioxidant capacity (TAC) of the media, percentage of DNA fragmentation, mitochondrial membrane potential (MMP), and metabolite quantification of the media by proton nuclear magnetic resonance (^{1}H-NMR). **Results:** The supplementation with VC (600 μM) and HYP (100 and 500 μM) did not induce

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any deleterious effects to the physiology and metabolism of the spermatozoa, after 1-hour of treatment. In the presence of H_2O_2 (300 μM), both VC and HYP were able to prevent some of the deleterious effects of H_2O_2 in sperm, which were represented by an increase in sperm motility, a decrease in DNA fragmentation, and a decreasing trend in lipid peroxidation levels. However, these antioxidants were not able to prevent the decrease of MMP associated with H_2O_2 treatment, nor were able to prevent the conversion of pyruvate into acetate (a reaction promoted by H_2O_2). ***Conclusion:*** The supplementation of sperm preservation media with VC and HYP could be beneficial for the preservation of sperm physiology. From the antioxidant conditions tested, the supplementation of media with HYP (100 μM) demonstrated the best results regarding sperm preservation, evidencing the higher antioxidant capacity of HYP compared to VC. Nevertheless, none of the antioxidants used was able to prevent the metabolic alterations promoted by H_2O_2 in spermatozoa.

Introduction

The decline in male fertility is an emerging problem in modern societies. The increasing trend for couples to have children at a later age, along with unhealthy lifestyles, and detrimental environmental factors are reducing the general fertility rates. According to the World Health Organization (WHO), infertility can be defined as a disease from the reproductive system that prevents a couple from conceiving, after 12 months of regular and unprotected sexual activity. This health issue affects approximately 15% of couples worldwide and is thought that the male factor is associated with around half of these cases [1]. Multiple causes affect male reproductive potential. However, about 30-50% of cases of male infertility have unexplained causes and are classified as idiopathic. Recently, it has been proposed that idiopathic infertility could be explained by the presence of high levels of reactive oxygen species (ROS) [2]. In fact, evidence suggests that ROS-mediated damage contributes to about 30-80% of male infertility cases [3].

In normal physiological conditions, reactive oxygen species (ROS) and antioxidants balance each other, which is essential for the maintenance of several molecular signalling pathways. However, when ROS exceeds the antioxidant capacity, the cell turns into a state of oxidative stress (OS). Prolonged exposure to OS is extremely prejudicial to cellular systems, promoting the disruption of signalling pathways, affecting essential metabolic processes, resulting in the degradation of cellular machinery [4]. The male reproductive system is very sensitive to OS. Spermatozoa are cells especially vulnerable to ROS-attack due to the abundance of polyunsaturated fatty acids (PUFAs) in their plasma membrane. PUFAs are essential for plasma membrane fluidity, which is essential for zona pellucida binding and the process of fertilization [5]. Furthermore, spermatozoa are known to carry a very limited amount of cytoplasm, which results in a lack of antioxidant defences, and inefficiency in repairing oxidative damage [6]. ROS are known for playing a dual role in biological systems, having prejudicial and beneficial effects. Evidence suggests that certain levels of ROS are required for physiological sperm functions, including sperm capacitation, hyperactivation, acrosomal reaction, and sperm-oocyte fusion [7-9]. On the other hand, in spermatozoa, the overproduction of ROS results in lipid peroxidation (LPO) of membranes [10], protein modification [11] DNA damage [12], and increased apoptosis. These pathological events have been negatively correlated with sperm motility [13], sperm morphology [14], and male fertility capacity [15].

Couples affected by infertility often choose to pursue parenthood through the help of assisted reproduction technology (ART). The implementation of ART allowed several infertile couples to achieve a successful pregnancy. However, the success rate of ART is still considered low, being at best 50.4 %, in < 35-year-old women with 3 good quality embryos, according to the 2011 study of Vernon, et al. [16]. ART is also known to increase the exposure of gametes and embryos to ROS. In the case of male gametes, the removal of seminal plasma, as well as sperm processing techniques (centrifugation, light exposure, temperature) causes

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2022;56(S1):1-23

DOI: 10.33594/000000487
Published online: 8 January 2022

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an increase in ROS production and promotes a reduction in sperm quality [17], decreasing (even more) the chances of achieving a successful pregnancy [18]. To prevent the oxidative damage of spermatozoa during ART, the sperm preparation medium is often supplemented with antioxidants.

Hyperoside (quercetin 3-O-β-D-galactoside) (HYP) is a flavonol glycoside, commonly found in plants of the genera *Hypericum* [19] and *Crataegus* [20]. It is a product of quercetin glycosylation of the hydroxyl group on C-3, with a galactoside group [21]. HYP extraction and isolation are difficult, due to their low concentrations, and their synthesis is costly. Nevertheless, this phytochemical has gained popularity due to its anti-cancer [22], anti-inflammatory [23], anti-thrombotic [24], and anti-depressant [25] pharmacological properties. Recent studies have also explored the antioxidant effects of HYP. HYP was reported to possess cytoprotective properties against OS in lung fibroblast cells [26]. Also, it was reported that HYP protects granulosa cells against hydrogen peroxide (H_2O_2)-induced cell apoptosis and OS, revealing potential benefits on female reproductive capacity [27]. Biagi M. and colleagues reported that *Castanea sativa* Miller leaf extract, particularly rich in HYP, showed scavenging properties against OS in human sperm and showed being capable to protect sperm membranes and the acrosomes [28]. All these findings suggest that HYP can be used as a potential antioxidant in disorders associated with oxidative damage. On the other hand, VC (L-ascorbic acid) is a water-soluble vitamin known for its antioxidant properties. This micronutrient is a reducing agent and acts like an electron donor that reacts with substrates such as H_2O_2, generating ascorbyl radicals, a relatively stable free radical [29]. VC cannot be synthesized by human metabolism, meaning that its uptake comes straight from food [30]. Its concentration in the seminal fluid can be 10 times higher than in the serum, with values ranging between 200-700 µM [31-35]. VC is commonly prescribed in oral antioxidant therapies for infertile men (usually in combination with other compounds), and it is reported to decrease sperm DNA fragmentation and improve sperm-quality parameters [36-39]. *In vitro* studies did also report that the supplementation of sperm medium with VC was able to decrease negative effects of vitrification on sperm parameters and chromatin quality [40], and improve DNA integrity [41, 42].

To our knowledge, no studies have been performed regarding the potential of HYP supplementation of the sperm preservation medium used during ART. Thus, we propose to test and compare the efficacy of sperm preservation media supplementation with HYP and VC in the prevention of spermatozoa-oxidative damage. To test the sperm-protective potential of these compounds, we supplemented Ham's F12 medium with VC (600 µM) and HYP (100 and 500 µM) and maintained spermatozoa for one hour at 37°C. Sperm motility and vitality, were evaluated before and after treatment. Subsequently, protein and lipid oxidative damage, DNA fragmentation, and mitochondrial membrane potential (MMP) of spermatozoa were assessed. The total antioxidant capacity (TAC) of the treatment media was also evaluated, and the spermatozoa metabolic profile was investigated.

Materials and Methods

Patient characterization and study design

Human sperm samples were collected from *Centro de Genética da Reprodução Professor Alberto Barros*, located in Porto, Portugal, after approval by the Joint Ethics Committee CHUP/ICBAS (2021/CE/P002[P342/CETI/ICBAS]) during November 2020 and June 2021. The semen samples were provided by male patients of reproductive age undergoing fertility assessment, after 2-4 days of abstinence. Semen samples were obtained by masturbation and placed in sterile tubes. Patients with inflammatory diseases, cancer, or other health conditions that may severely affect their fertility potential were discarded. Also, patients were asked to provide information on the consumption of tobacco, alcohol, and other substances that could interfere with the experiment. All patients included in this study signed informed written consent. Sperm samples were characterized according to the WHO guidelines for laboratory examination and processing of human sperm [43]. Only samples having the following normal semen parameters, according to the WHO-criteria,

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2022;56(S1):1-23

DOI: 10.33594/000000487
Published online: 8 January 2022

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were used in this project: sperm concentration ≥ 15 million/mL, total sperm motility ≥ 40%, and sperm vitality ≥ 58%. The fresh semen samples were centrifuged at 500 *g* for 5 minutes at room temperature. The seminal fluid was discarded, and the pellet was washed with 1 mL of phosphate-buffered saline (PBS) solution. After another centrifugation at 500 *g* for 5 minutes, the supernatant was discarded, and the pellet was resuspended in Ham F12 medium.

To test if HYP (HWI group, Rülzheim, Germany) had a cytotoxic effect in human spermatozoa, sperm samples (10 million sperm/condition) were incubated with increasing concentrations of HYP (0, 5, 50, 100, and 500 μM), for 1h at 37°C, 5% CO_2. Sperm vitality and sperm motility were measured before and after the experiment. The concentrations of 100 and 500 μM of HYP (1h of incubation time) were selected for further experiments. To test the sperm-protective potential of HYP and VC (Sigma-Aldrich, St. Louis, Missouri, USA), 10 million spermatozoa were incubated for 1h at 37°C, 5% CO_2 in the following conditions: 1) Ham F12 medium; 2) Ham F12 medium + 600 μM VC; 3) Ham F12 medium + 100 μM HYP; 4) Ham F12 medium + 500 μM HYP; 5) Ham F12 medium + 300 μM H_2O_2; 6) Ham F12 medium + 600 μM VC + 300 μM H_2O_2; 7) Ham F12 medium + 100 μM HYP + 300 μM H_2O_2; 8) Ham F12 medium + 500 μM HYP + 300 μM H_2O_2. H_2O_2 (LabChem, Lisbon, Portugal) was used as a positive control for OS, as described in [44, 45]. VC concentration of 600 μM was chosen according to [31-35]. Sperm vitality and sperm motility were assessed before and after the experiment according to the WHO guidelines [43]. Afterwards, samples were centrifuged at 500 *g*, 37°C. Sperm pellets and *post*-treatment media were stored at -80°C until used in further analysis.

Oxidative stress evaluation

Sperm pellets were resuspended in 10 μL of 1% SDS lysis buffer (1 μL for every 10^6 cells). The samples were vortex and left at room temperature for 60 min to promote the lysis reaction. Subsequently, lysates were centrifuged at 14000 *g* for 20 minutes, at room temperature. The cell debris pellet was discarded. Protein quantification was performed using the Pierce Bicinchoninic acid protein assay (Thermo Fisher Scientific, Massachusetts, USA). Levels of lipid peroxidation, protein carbonylation, and protein nitration were evaluated by immunoblotting. For lipid peroxidation and protein nitration levels detection, 5 μg of protein was diluted in PBS to a final volume of 100 μL and transferred to polyvinylidene difluoride (PVDF) membranes through a slot-blot system. For protein carbonylation evaluation, we used the protocol described in [46]. In resume, 5 μg of protein was diluted in PBS to a final volume of 20 μL. To each sample was added 20 μL of 12% SDS solution for protein denaturation and carbonyl groups exposer. Subsequently, 40 μL of 20 mM 2,4-dinitrophenylhydrazine (DNPH) in 10% trifluoroacetic acid (TFA) was added, and the reaction was allowed to occur for 30 min at room temperature, in the dark. To stop the reaction between carbonyl groups and DNPH, 30 μL of 2M Tris with 18% β-mercaptoethanol solution was added. 2.4 μL of the derivatized samples were diluted in 107.6 μL of PBS and transferred to PVDF membranes through a slot-blot system.

Membranes were then blocked with a blocking solution [5% bovine serum albumin (BSA) in Tris-Buffered Saline, 0.1% Tween 20 (TBS-T)] for 1h30 and incubated overnight with the correspondent primary antibody. For lipid peroxidation evaluation, the primary antibody used was anti-4-hydroxynonenal (4-HNE) (1:1000 diluted in 1% BSA TBS-T; AB5605, EMD Millipore, Temecula, CA, USA). For protein nitration evaluation, the primary antibody used was anti-3-nitrotyrosine (NT) (1:1000 diluted in 1% BSA TBS-T; 9691S, Cell Signaling Technology, Danvers, MA, USA). For protein carbonylation evaluation, the primary antibody used was anti-dinitrophenol (DNP) (1:5000 diluted in 1% BSA TBS-T; D9656, Sigma-Aldrich, St. Louis, Missouri, USA). Membranes were washed with TBS-T and incubated with an appropriate secondary antibody for 1h30 [Anti-Goat 1:5000 diluted in 1% BSA TBS-T (A4187) or Anti-Rabbit 1:5000 diluted in 1% BSA TBS-T (A3687), both from Sigma-Aldrich, St. Louis, Missouri, USA]. Immuno-reactive proteins were detected through reaction with an ECF substrate (GE Healthcare, Buckinghamshire, UK), and analyzed by ChemiDoc™ MP Imaging system (Bio-Rad, California, USA). Total protein was assessed and quantified by Ponceau staining. Results were normalized to total protein.

Post-treatment media total antioxidant capacity

The TAC of the *post*-treatment media samples was measured by Ferric Reducing Antioxidant Power (FRAP) assay as described in [47]. FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1.

Briefly, in a 96-well plate, 6 μL of *post*-treatment media samples were added to 180 μL of FRAP reagent. Triplicates were made for each sample. Distilled water (dH_2O) was used as negative control and VC (1000 μM)

was used as an antioxidant standard. Both controls were treated in the same way as media samples. The absorbance was read at 593 nm, immediately after the addition of the FRAP reagent (0 min) and after 40 min. Between measurements, the plate was kept in the dark, at room temperature. The antioxidant potential of the samples was determined against VC (1000 µM) standard and corrected using the absorbance value of dH$_2$O absorbance. FRAP value (µmol antioxidant potential/L) was calculated using the formula described in [47].

Sperm DNA fragmentation evaluation

The evaluation of sperm DNA fragmentation was performed by the terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay, using in-situ Cell Death Detection Kit (Fluorescein) from Roche Diagnostics GmbH (Mannheim, Germany). Spermatozoa (10 million) were collected and treated with HYP, and VC as previously described. A smear of each sample was performed on adhesive slides. The slides were subsequently fixed with 4% paraformaldehyde, for 1h at room temperature and then rinsed with PBS. For cell permeabilization, slides were immersed in a solution of 0.1% sodium citrate containing 0.1% Triton X, for 2 min at 4°C. After two washes with PBS for 5 min, 50 µL of the TUNEL reaction mix [5 µL of terminal deoxynucleotidyl transferase (TdT) and 45 µL of labeled deoxyuridine triphosphate nucleotides (dUTPs) mixture] was added to each slide. A coverslip was placed to prevent evaporation. The slides were incubated in a humidified chamber at 37°C for 1h, in darkness. The slides were then washed four times with PBS (2 min each). Subsequently, coverslips were mounted using mounting medium VECTASHELD (30 Ingold Road, Burlingame, CA 94010, USA). The results were visualized in a Nikon Eclipse E400 microscope equipped with a Y-FL epi-fluorescence attachment and HB-10103AF Super high-pressure mercury lamp power supply (Nikon, Shinagawa, Tokyo, Japan), coupled with a Nikon NIS Elements Image Software. 200 spermatozoa were counted on each slide. The total number of spermatozoa was quantified under blue fluorescence (corresponding to the DAPI staining of the nucleus). Spermatozoa presenting DNA fragmentation also present green fluorescence (corresponding to the dUTPs inserted in the DNA breaks by TdT). The percentage of DNA fragmentation was calculated.

Mitochondrial membrane potential evaluation

The MMP of spermatozoa was measured by using the lipophilic probe 5-5′,6-6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (T3168, Invitrogen™, Carlsbad, CA, USA), which can selectively penetrate mitochondria [45]. In spermatozoa with damaged mitochondrial membrane, the JC-1 remains in the cytoplasm, in a monomeric form. Meanwhile, in spermatozoa with normal mitochondrial membranes occur the accumulation of JC-1 in the membrane, promoting the formation of JC-1 aggregates. Briefly, 1 million spermatozoa were collected after treatment, washed with PBS (500 g, 5 min) and incubated with JC-1 (1 µg/mL in PBS) for 30 min at 37°C. As a positive control, spermatozoa were incubated with a 20% DMSO solution to promote the increase of mitochondrial membrane potential. After the incubation period, spermatozoa were washed with PBS (500 g, 5 min) two times. Pellets were resuspended in 750 µL of PBS and transferred to 96-well plates (performed in triplicate). The fluorescence of the JC-1 monomers (ex 485/530 nm; excitation/emission) and JC-1 aggregates (535/590 nm; excitation/emission) were assessed by a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). The ratio between JC-1 aggregates/monomers was calculated and used as an MMP marker.

Post-treatment media metabolite analysis

Metabolites present in the *post*-treatment media were assessed by proton nuclear magnetic resonance (^{1}H-NMR) and spectra analysis. 1D ^{1}H-NMR spectra were acquired on a 500MHz Bruker Avance III HD spectrometer equipped with a 5-mm TXI probe, at 298K. To each *post*-treatment medium (180 µL) it was added 45 µL of sodium fumarate (final concentration = 2 mM), for a final sample volume of 225 µL. Sodium fumarate was used as an internal reference for metabolite quantification in the media. The quantified metabolites were the following (multiplicity, chemical shift(ppm)): pyruvate (singlet, 2.37), acetate (singlet, 1.90), lactate (doublet, 1.32), alanine (doublet, 1.47), and malate (double doublet, 2.65). The spectra were manually phased, and baseline corrected. NUTS-Pro™ NMR software (Acorn NMR, Inc, Fremont, CA, USA) was used to integrate the chosen metabolite peaks. The concentration of the metabolites is expressed as nmol/10^6 sperm cells.

Statistical Analysis

All data presented are expressed as whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). Variations between groups regarding sperm total motility, sperm vitality, OS biomarkers, DNA fragmentation, MMP, and metabolite quantification were evaluated by one-way ANOVA followed by the correction for multiple comparisons by controlling the False Discovery Rate (FDR). The two-stage step-up method of Benjamini, Krieger, and Yekutieli was the test used to assess multiple comparisons. Fold variation to the 0 µM group was calculated for OS biomarkers quantification, DNA fragmentation evaluation, and MMP assessment. The differences between the groups in the cytotoxic test and the TAC media assessment were performed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. Values of $P < 0.05$ were considered statistically different. The statistical analysis of this work was performed by GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA).
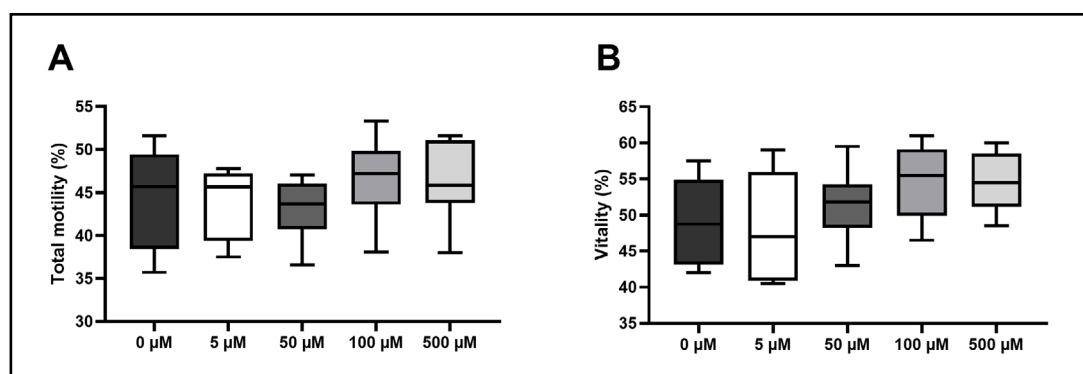
Results

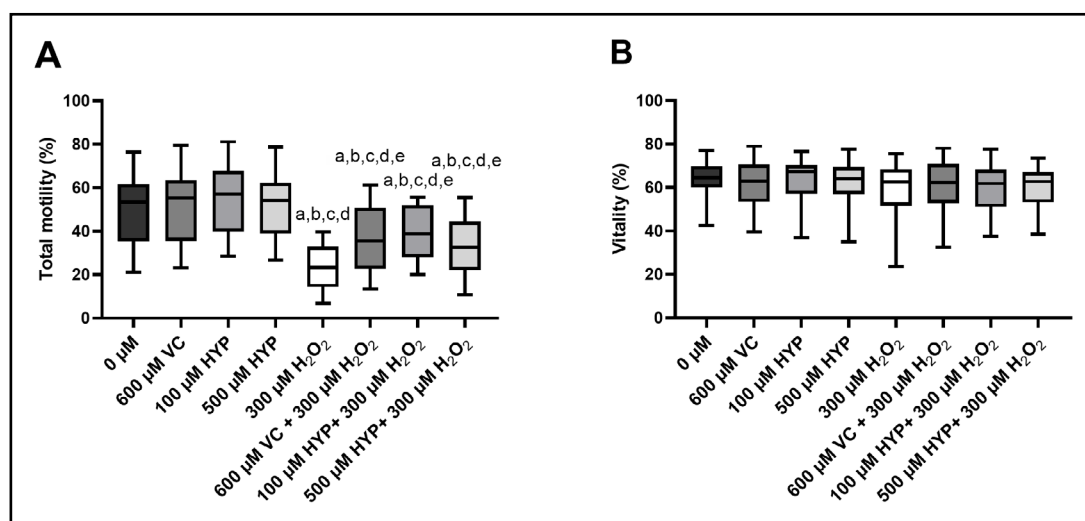### Supplementation of sperm preservation media with hyperoside has no cytotoxic effects on human sperm

After treating human spermatozoa with increasing doses of HYP (in µM: 0, 5, 50, 100, and 500), for 1h at 37°C, cytotoxicity was evaluated as in [48, 49], through assessment of sperm motility and vitality. Fig. 1 represents sperm total motility and sperm vitality, after preservation in media supplemented with HYP. Regarding sperm motility, we could not find any significant differences between the HYP treated groups (in µM: 5, 50, 100, and 500), and the control group (0 µM) (Fig. 1A). Similarly, we could not find any significant differences in spermatozoa vitality after preservation in media supplemented with HYP at any concentration, and the control group (Fig. 1B). No cytotoxic effects were found in sperm samples preserved in media supplemented with any of the tested HYP concentrations. The concentrations of 100 and 500 µM of HYP were selected for further testing.

Supplementation of sperm preservation media with vitamin C and hyperoside preserves sperm motility under oxidative stress conditions

To test the cytoprotective effects of VC and HYP supplementation in human spermatozoa the following concentration were selected: 600 µM for VC [31-35]; 100 and 500 µM for HYP (after considering the previous cytotoxicity test results), 1h at 37°C. H_2O_2 (300 µM) was used as an OS-inducer in spermatozoa. Fig. 2 represents spermatozoa total motility and

Fig. 1. Evaluation of hyperoside (HYP) cytotoxicity for human spermatozoa. (A) represents the percentage of sperm total motility (N=6) after supplementation with increasing doses of HYP. (B) represents the percentage of sperm vitality (N=6) after supplementation with increasing doses of HYP. Results are represented as Tukey's whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). No statistical differences were found between groups (one-way ANOVA followed Tukey post hoc test for multiple comparisons).

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2022;56(S1):1-23

DOI: 10.33594/000000487
Published online: 8 January 2022

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Fig. 2. Evaluation of the cytoprotective effects of vitamin C (VC) and hyperoside (HYP) sperm media supplementation under oxidative stress conditions. (A) represents the variations in the percentage of total sperm motility (N=20) after 1h treatment with VC (600 μM), and HYP (100 and 500 μM) in the presence and absence of H_2O_2 (300 μM), herein used as an OS inducer. (B) represents the variations in the percentage of sperm vitality under the same conditions (N=20). Results are represented as whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). Significantly different results (one-way ANOVA followed by the correction for multiple comparisons by controlling FDR, two-stage step-up method of Benjamini, Krieger, and Yekutieli; $p < 0.05$) are indicated as (a) relative to control; (b) relative to 600 μM VC group; (c) relative to 100 μM HYP group; (d) relative to 500 μM HYP group; (e) relative to 300 μM H_2O_2 group.

spermatozoa vitality, after preservation in media supplemented with VC and HYP, in the presence and absence of H_2O_2. We found no differences regarding spermatozoa total motility after preservation in media supplemented with VC or HYP, in comparison with the control group (0 μM), illustrating that supplementation of media with these antioxidants does not affect spermatozoa motility (Fig. 2A). However, spermatozoa motility decreased in the group exposed to H_2O_2 (300 μM) (22 ± 10 %) in comparison to the control group (50 ± 15 %) and those preserved in media supplemented with VC and HYP (100 and 500 μM) (52 ± 16 %; 55 ± 15 % and 51 ± 14 %, respectively) suggesting that spermatozoa are under stress (Fig. 2A). The spermatozoa motility of the group preserved in the media supplemented with VC and treated with H_2O_2 (36 ± 15 %) was higher than in the group treated with H_2O_2 alone suggesting a cytoprotective effect of VC supplementation against the damage promoted by H_2O_2. Nevertheless, the spermatozoa motility of this group was decreased in comparison to the control group and the only-antioxidant groups. The motility of the groups preserved in media supplemented with HYP (100 and 500 μM) and treated with H_2O_2 (39 ± 12 % and 35 ± 13 %, respectively) was higher than the motility of the group treated only with H_2O_2. Still, the spermatozoa motility of those groups decreased when compared to the control group and the only-antioxidant groups. These results suggest a protective effect of the antioxidants VC and HYP under OS conditions.

Regarding spermatozoa vitality, none of the preservation media supplemented with VC and HYP (100 and 500 μM) with or without H_2O_2 promoted a decrease of spermatozoa vitality after 1h treatment (Fig. 2B). These results suggest that, although the concentration of 300 μM of H_2O_2 can induce a decrease in sperm motility, this treatment does not affect sperm vitality.

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2022;56(S1):1-23

DOI: 10.33594/000000487
Published online: 8 January 2022

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Supplementation of sperm preservation media with hyperoside increases the total antioxidant capacity of the media

After preserving spermatozoa with media supplemented with HYP (100 and 500 μM) in the presence and absence of H_2O_2 for 1h at 37°C, TAC of the media was measured by FRAP. Fig. 3A represents the TAC levels of the media, herein measured by FRAP levels. VC was used as a standard for antioxidant capacity, and, because of that, TAC of the sperm preservation media supplemented with VC (600 μM) was not assessed. Nevertheless, VC (1000 μM) was used as a standard value of TAC (2 μmol antioxidant capacity/L). The media containing 500 μM of HYP (in the presence and absence of H_2O_2) presented the highest TAC levels (4.78 ± 0.73 and 4.54 ± 0.58 μmol antioxidant capacity/L, respectively). Also, the TAC value of the spermatozoa preservation media supplemented with 500 μM of HYP was higher than the standard TAC level of VC (1000 μM). The media containing 100 μM of HYP (in the presence and absence of H_2O_2) also presented higher TAC levels (1.22 ± 0.31 and 1.15 ± 0.24 μmol antioxidant capacity/L, respectively) in comparison to the control (0.21 ± 0.06 μmol antioxidant capacity/L) and only-H_2O_2 group (0.18 ± 0.06 μmol antioxidant capacity/L). Although the TAC of these media was lower than the ones containing 500 μM of HYP. Additionally, the TAC of the spermatozoa preservation media supplemented with 100 μM of HYP was lower than the standard TAC level of VC (1000 μM). The medium containing H_2O_2 (300 μM) had the lowest TAC from all the media tested. However, no difference was found regarding the TAC from this group and the TAC from the control group (0 μM). Notwithstanding, the FRAP does not react with H_2O_2, which can justify the absence of differences concerning the groups with the presence of H_2O_2 [50]. The FRAP assay measures the ability of an antioxidant compound to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) and this reaction is linked to a colour change (absorbance at 593 nm) [47].

Supplementation of sperm preservation media with vitamin C and hyperoside appears to protect human sperm against lipid peroxidation under oxidative stress conditions
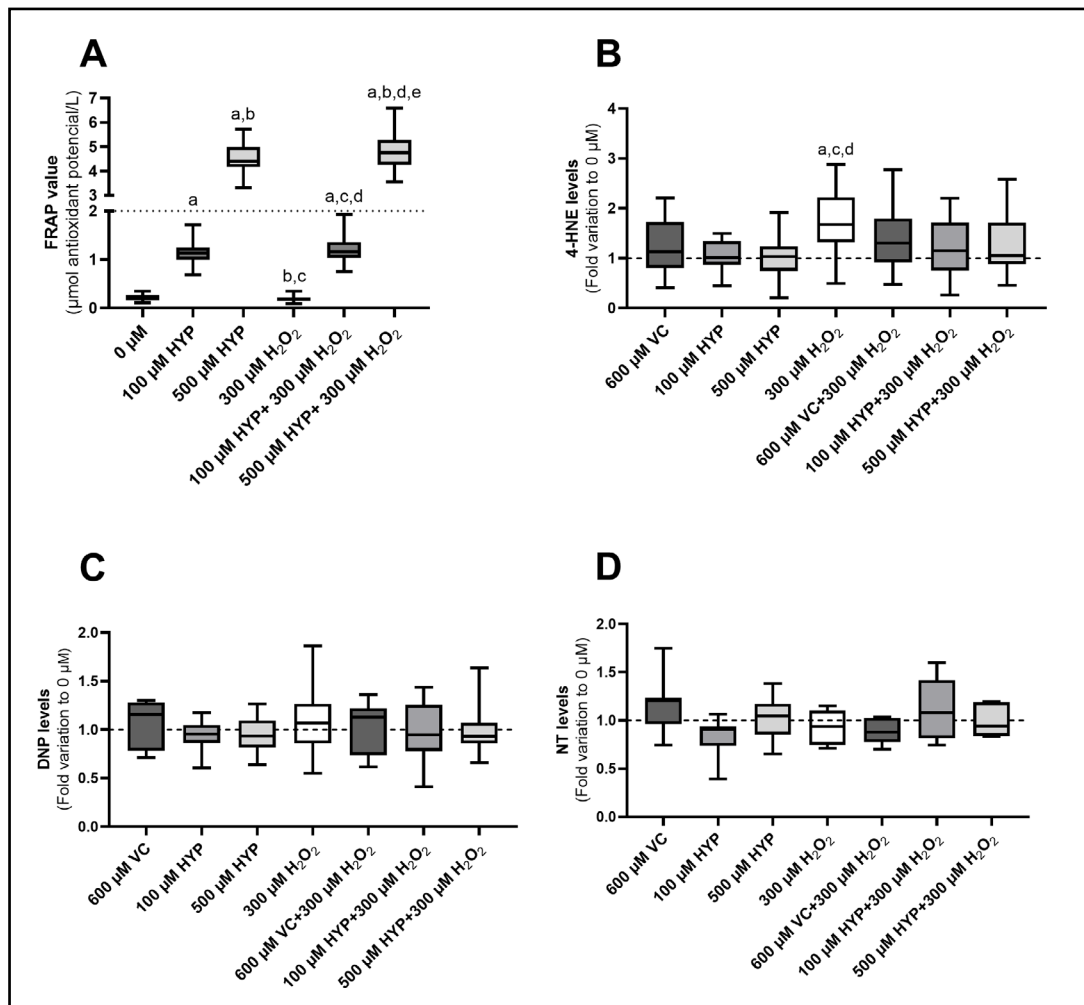
Elevated levels of H_2O_2 are known to promote OS and cause several damages to spermatozoa biomolecules, such as lipids and proteins. OS biomarkers were quantified to evaluate the oxidative damage promoted by H_2O_2 in human sperm and to further test the cytoprotective effects of sperm preservation media supplementation with VC and HYP. Fig. 3 (panels B, C, and D) represents the levels of lipid peroxidation, protein carbonylation, and protein nitration found in human sperm after supplementation of sperm preservation media with VC and HYP in the presence and absence of H_2O_2, 1h, 37°C.

Lipid peroxidation was assessed by the quantification of 4-HNE levels (Fig. 3B). Lipid peroxidation levels of the groups preserved in media supplemented with VC and HYP (100 and 500 μM) (1.25 ± 0.54; 1.04 ± 0.31 and 1.04 ± 0.45 fold, respectively) and the control group (represented as a dashed line, treatment with no HYP, no VC, and no H_2O_2) were similar, after 1h treatment. Meanwhile, in the group treated with only H_2O_2 (300 μM) (1.71 ± 0.70 fold) the spermatozoa lipid peroxidation levels increased when compared to the control group and those preserved in media supplemented with HYP (100 and 500 μM) groups. The peroxidation levels of spermatozoa preserved in media supplemented with VC and HYP (100 and 500 μM), in the presence of H_2O_2 (1.39 ± 0.61; 1.23 ± 0.58 and 1.30 ± 0.57 fold, respectively) were not significantly different from the levels detected in spermatozoa from the group treated with H_2O_2 alone (Fig. 3B). Notwithstanding, spermatozoa peroxidation levels in those groups tend to be lower in comparison to the levels detected in spermatozoa subjected only to H_2O_2, suggesting that cytoprotective action of VC and HYP may be occurring to some extent.

The DNP was used to assess protein carbonylation levels induced by our treatments in spermatozoa (Fig. 3C). No statistical differences were found regarding the carbonylation levels of spermatozoa preserved in media supplemented with VC and HYP (100 and 500 μM) in the presence and absence of H_2O_2 in comparison to the control group. Further, no statistical difference was found regarding spermatozoa carbonylation levels after treatment with H_2O_2 alone, and the control group (Fig. 3C). Similar results were found regarding spermatozoa

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2022;56(S1):1-23

DOI: 10.33594/000000487
Published online: 8 January 2022

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protein nitration levels, evaluated by the quantification of NT (Fig. 3D). No statistical differences were found regarding the nitration levels of spermatozoa preserved in media supplemented with VC and HYP (100 and 500 µM) in the presence and absence of H_2O_2 in comparison to the control group. Similarly, no statistical differences were found regarding the nitration levels of the groups treated with H_2O_2 (300 µM), and the control group (Fig. 3D).

Fig. 3. Effects of vitamin C (VC) and hyperoside (HYP) media supplementation, under oxidative stress conditions, in the total antioxidant capacity (TAC) of the media and quantification of oxidative stress biomarkers in preserved spermatozoa. (A) represents the antioxidant capacity of media (N=20). Significantly different results (one-way ANOVA followed Tukey post hoc test for multiple comparisons; $p<0.05$) are indicated as (a) relative to control; (b) relative to 100 µM HYP group; (c) relative to 500 µM HYP group; (d) relative to 300 µM H_2O_2 group; (e) relative to 100 µM HYP + 300 µM H_2O_2 group. (B) represents the 4-HNE expression levels found in spermatozoa after 1h treatment in media supplemented with VC (600 µM), and HYP (100 and 500 µM) in the presence and absence of H_2O_2 (300 µM), herein used as an oxidative stress inducer (N=20). (C) represents DNP levels quantified after the reaction of DNPH with spermatozoa's protein carbonyl groups, after treatments (N=10). (D) represents the NT expression levels found in spermatozoa, after treatments (N=10). Fold variation was performed to the control group (Ham-F12 medium with no HYP, no VC, nor H_2O_2) represented as a dashed line. Significantly different results (one-way ANOVA followed by the correction for multiple comparisons by controlling FDR, two-stage step-up method of Benjamini, Krieger, and Yekutieli; $p<0.05$) are indicated as (a) relative to control; (c) relative to 100 µM HYP group; (d) relative to 500 µM HYP group. All results are represented as Tukey's whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values).
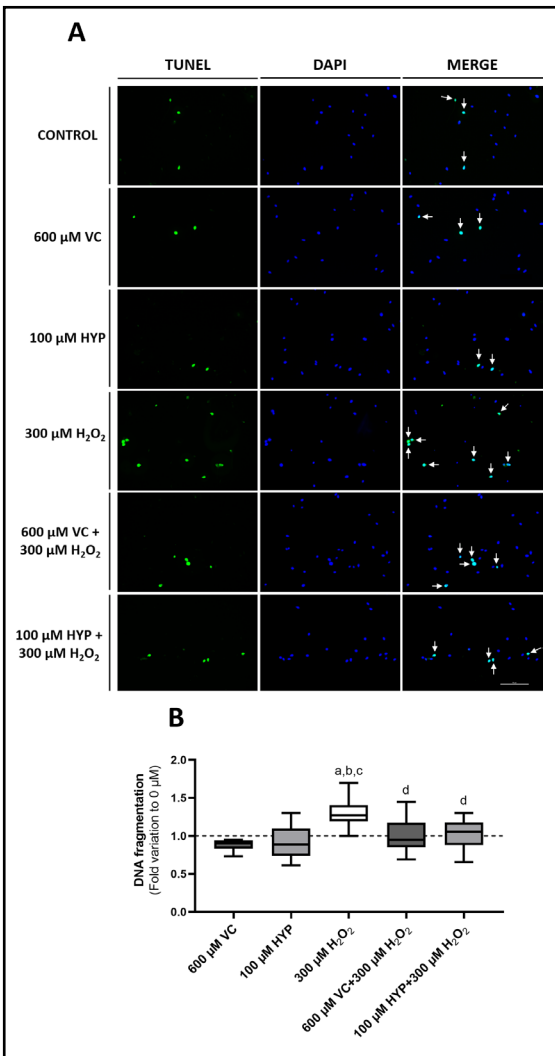
Supplementation of sperm preservation media with vitamin C and hyperoside prevents DNA fragmentation in human spermatozoa under oxidative stress conditions

One of the consequences of a prolonged OS situation is DNA damage [12], and increased apoptosis. In spermatozoa, DNA damage is known to severely decrease the chances of achieving a successful pregnancy and increase the risk of abortion. To test if supplementation of sperm media with VC and HYP was able to prevent DNA fragmentation induced by OS we performed a TUNEL assay after preservation in the experimental conditions. Fig. 4A represents the result of the TUNEL assay visualized by fluorescence. Spermatozoa with DNA fragmentation present a green fluorescence corresponding to the dUTPs inserted in the DNA breaks. The nuclei were stained with DAPI, which has a blue fluorescence. The merge of the green/blue channels allows the detection of the percentage of sperm DNA fragmentation for each condition. Fig. 4B represents the percentage of DNA fragmentation detected in human spermatozoa after 1h incubation in media supplemented with VC and HYP (100 μM) in the presence and absence of H_2O_2. Our results showed no differences regarding sperm DNA fragmentation between the control group (represented as a dashed line, treatment with no HYP, no VC, and no H_2O_2), the VC (600 μM) (0.88±0.08-fold) and the HYP (100 μM) (0.91±0.22-fold) supplemented groups. This indicates that the supplementation with VC (600 μM) and HYP (100 μM) does not induce DNA fragmentation *per se.* Spermatozoa treated with H_2O_2 (300 μM) had higher levels of DNA fragmentation by a 1.30 ± 0.19 fold variation to the control group. Furthermore, we found that spermatozoa supplemented with VC and HYP (100 μM) in the presence of H_2O_2 (1.02 ± 0.23 and 1.02 ± 0.20 fold, respectively) exhibited lower levels of DNA fragmentation compared to the group treated only with H_2O_2. In addition, the DNA fragmentation levels of these groups did not differ from the control and antioxidants-only groups. These results may suggest that a cytoprotective effect against DNA damage could be promoted by the supplementation of VC and HYP in sperm preservation media.
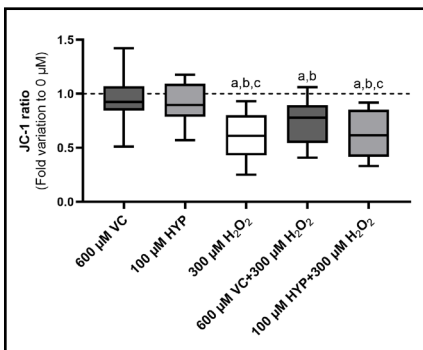
Supplementation of sperm preservation media with vitamin C and hyperoside cannot prevent alteration in the sperm mitochondrial membrane under oxidative stress conditions

MMP is a strong indicator of mitochondrial functionality and can be used as a measure of electron transport chain (ETC) activity. Mitochondria are fundamental to producing cellular energy. In spermatozoa, several studies are reporting a positive correlation between MMP and motility. To test if media supplementation with VC and HYP can ensure mitochondrial activity, under conditions of OS, we used the JC-1 probe. Fig. 5 represents the ratio of JC-1-aggregates/monomers in human spermatozoa, after 1h preservation in media supplemented with VC (600 μM) and HYP (100 μM) in the presence and absence of H_2O_2. Our results show no differences between the groups preserved in media supplemented with VC (600 μM) (0.97 ± 0.23 fold) and HYP (100 μM) (0.92 ± 0.19 fold) and the control group (represented as a dashed line, treatment with no HYP, no VC, and no H_2O_2), meaning that no MMP alterations were promoted by the antioxidant treatment, *per se*. A decrease in the MMP of spermatozoa can be observed in the group treated with H_2O_2 (300 μM) in comparison with the control group and with those preserved in media supplemented with antioxidants. However, no differences were found regarding spermatozoa MMP between the groups preserved in media supplemented with VC and HYP (100 μM), in the presence of H_2O_2, (0.72 ± 0.20 and 0.64 ± 0.21 fold, respectively), and the group treated with H_2O_2-only (0.59 ± 0.22 fold). Also, following sperm motility results (Fig. 2A), the MMP of the groups preserved in media supplemented with antioxidants treated with H_2O_2 was decreased when compared to MMP of spermatozoa from the control and antioxidants-only groups. This suggests that the presence of VC (600 μM) and HYP (100 μM) in the sperm media is not able to prevent the decrease in the sperm MMP promoted by H_2O_2.

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Fig. 4. Effects of vitamin C (VC) and hyperoside (HYP) media supplementation in spermatozoa DNA fragmentation under oxidative stress conditions. (A) represents the results of immunofluorescence staining of the spermatozoa DNA breaks by TUNEL (ex 480/535 nm; excitation/emission), and spermatozoa nuclei by DAPI (ex 395/460nm; excitation/emission). (B) represents the percentage of DNA fragmentation quantified in spermatozoa after 1h treatment in media supplemented with VC (600 μM), and HYP (100 μM) in the presence and absence of H_2O_2 (300 μM), herein used as an oxidative stress inducer (N=10). Fold variation was performed to the control group (Ham-F12 medium with no HYP, no VC, nor H_2O_2) represented as a dashed line. Results are represented as Tukey's whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). Significantly different results (one-way ANOVA followed by the correction for multiple comparisons by controlling FDR, two-stage step-up method of Benjamini, Krieger, and Yekutieli; $p < 0.05$) are indicated as (a) relative to control; (b) relative to 600 μM VC group; (c) relative to 100 μM HYP group; (d) relative to 300 μM H_2O_2 group.

Fig. 5. Effects of vitamin C (VC) and hyperoside (HYP) media supplementation in mitochondria functionality of spermatozoa under oxidative stress conditions. Fold variation was performed to the control group (Ham-F12 medium with no HYP, no VC, nor H_2O_2) represented as a dashed line. Results are represented as Tukey's whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). Significantly different results (one-way ANOVA followed by the correction for multiple comparisons by controlling FDR, two-stage step-up method of Benjamini, Krieger, and Yekutieli; $p < 0.05$) are indicated as (a) relative to control; (b) relative to 600 μM VC group; (c) relative to 100 μM HYP group.

Supplementation of sperm preservation media with vitamin C and hyperoside cannot prevent alterations in the metabolism of spermatozoa under oxidative stress conditions

Metabolism is a network of biochemical reactions that ensure cell function. The study of spermatozoa metabolism can enlighten concerning what is going inside the cell. The effects of VC (600 μM) and HYP (100 and 500 μM) supplementation, in the presence and absence of H_2O_2, on spermatozoa metabolism were assessed by ^{1}H-NMR spectra analysis of the media. Fig. 6 represents the main metabolites identified in the studied groups: pyruvate (Fig. 6A); acetate (Fig. 6B); lactate (Fig. 6C); alanine (Fig. 6D) and malate (Fig. 6E).
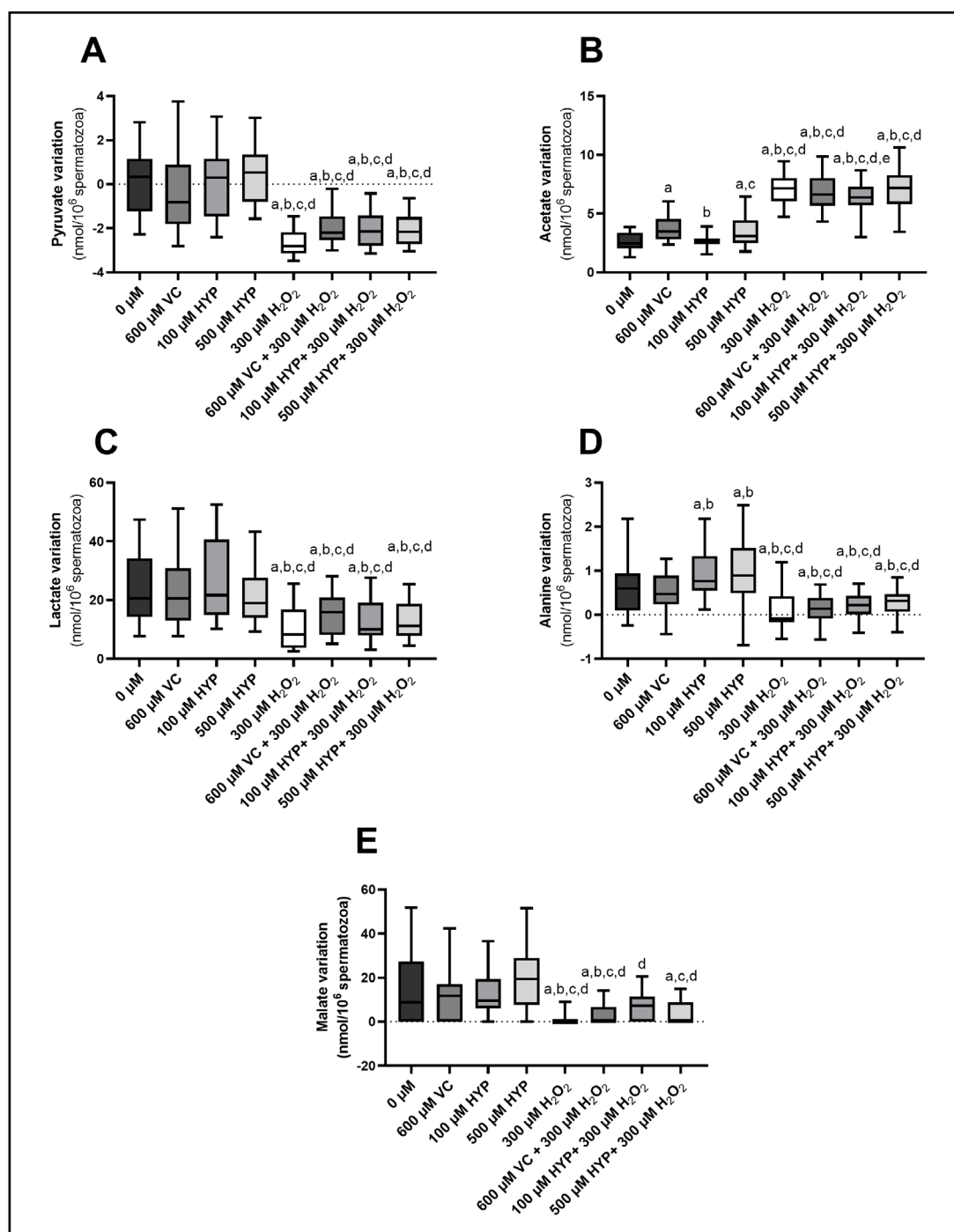
Concerning pyruvate variation, no differences were observed in pyruvate variation in the media, between the control (0.09 ± 1.58 nmol/10^6 cells) and the ones supplemented with VC (600 μM), and HYP (100 and 500 μM) (-0.23 ± 1.92; 0.06 ± 1.58 and 0.65 ± 1.48 nmol/10^6 cells, respectively) (Fig. 6A). The group treated with H_2O_2 presented the lowest levels of pyruvate (-2.65 ± 0.57 nmol/10^6 cells). The media supplemented with VC (600 μM), and HYP (100 and 500 μM), in the presence of H_2O_2, (-2.00 ± 0.83; -2.11 ± 0.81 and -2.08 ± 0.74 nmol/10^6 cells, respectively) presented similar pyruvate levels to the H_2O_2-only group, which were significantly lower when compared to the control group and the antioxidants-only groups (Fig. 6A).

Regarding acetate media levels, we found that media supplementation with VC (600 μM) (3.72 ± 1.09 nmol/10^6 cells) and HYP (500 μM) (3.49 ± 1.27 nmol/10^6 cells), presented an increase in acetate production by spermatozoa compared to the control group (2.65 ± 0.76 nmol/10^6 cells). However, no differences were found between the acetate levels of the HYP (100 μM) group (2.66 ± 0.56 nmol/10^6 cells) and the control group. On the other hand, we observed higher levels of acetate in the medium of spermatozoa treated only with H_2O_2 (7.11 ± 1.25 nmol/10^6 cells) (Fig. 6B). The levels of acetate in spermatozoa from VC (600 μM) plus H_2O_2 group (6.80 ± 1.52 nmol/10^6 cells) and the HYP (500 μM) plus H_2O_2 group (7.04 ± 1.59 nmol/10^6 cells) were similar to the acetate levels of the H_2O_2-only group. Nevertheless, we also ascertain that acetate levels in the media of spermatozoa preserved in media supplemented with HYP (100 μM) plus H_2O_2 (6.35 ± 1.51 nmol/10^6 cells) were lower than in the only-H_2O_2 group (Fig. 6B).

Concerning lactate media levels, no differences were found regarding the lactate levels of the control group (24.02 ± 12.43 nmol/10^6 cells), the ones supplemented with VC (600 μM), and HYP (100 and 500 μM) (22.36 ± 11.85; 26.11 ± 14.05 and 21.67 ± 10.10 nmol/10^6 cells, respectively). Lactate production by spermatozoa was decreased in the group treated with only H_2O_2 (10.79 ± 7.23 nmol/10^6 cells) in comparison with control and the antioxidant-only groups. In addition, the levels of lactate of the media supplemented with VC (600 μM) and the HYP (100 and 500 μM), in the presence of H_2O_2 (15.28 ± 7.07; 13.12 ± 7.17 and 13.14 ± 6.46 nmol/10^6 cells, respectively) were similar to the lactate levels of the H_2O_2-only group (Fig. 6C) and lower when compared to the control group and the antioxidants-only groups.

Interestingly, the media of spermatozoa preserved with HYP (100 and 500 μM) presented higher levels of alanine (0.93 ± 0.60 and 1.00 ± 0.77 nmol/10^6 cells, respectively) when compared with the control group (0.61 ± 0.65 nmol/10^6 cells), and the one supplemented with VC (600 μM) (0.53 ± 0.44 nmol/10^6 cells). As seen in other metabolites, the media with only H_2O_2 supplementation (0.12 ± 0.48 nmol/10^6 cells), and the media supplemented with VC (600 μM) and HYP (100 and 500 μM), in the presence of H_2O_2 (0.13 ± 0.31; 0.20 ± 0.31 and 0.25 ± 0.34 nmol/10^6 cells, respectively) presented decreased alanine levels when compared to the control and antioxidants-only groups (Fig. 6D).

Concerning malate variation, no differences were found in media malate levels between the control group (14.36 ± 17.04 nmol/10^6 cells) and the ones supplemented with VC (600 μM), and HYP (100 and 500 μM) (11.63 ± 11.39; 12.95 ± 11.56 and 20.00 ± 15.63 nmol/10^6 cells). The H_2O_2-only group presented the lowest malate levels (1.25 ± 2.76 nmol/10^6 cells) in comparison to the control and the antioxidant-only groups. Decreased malate levels were also in media supplemented with VC (600 μM) and HYP (100 and 500 μM), in the presence of H_2O_2, (2.71 ± 4.66; 7.21 ± 6.02 and 4.33 ± 5.46 nmol/10^6 cells, respectively) (Fig. 6E).

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Fig. 6. Effects of vitamin C (VC) and hyperoside (HYP) media supplementation under oxidative stress conditions, in pyruvate (A), acetate (B), lactate (C), alanine (D), and malate (E) levels found in the sperm preservation media. All results are represented as Tukey's whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). Significantly different results (one-way ANOVA followed by the correction for multiple comparisons by controlling FDR, two-stage step-up method of Benjamini, Krieger, and Yekutieli; $p < 0.05$) are indicated as (a) relative to control (0 μM); (b) relative to 600 μM VC group; (c) relative to 100 μM HYP group; (d) relative to 500 μM HYP group; (e) relative to 300 μM H_2O_2 group.

Discussion

ROS are part of the complex signalling network used by cells. In spermatozoa, ROS participate in several physiological events such as capacitation, hyperactivation, acrosomal reaction, and sperm-oocyte fusion [7-9]. These events are essential for sperm function and to ensure a successful pregnancy. Nevertheless, the overproduction of ROS is responsible for the increase of deleterious events such as lipid peroxidation [10] (that severely impacts sperm membranes), protein modifications [11], DNA fragmentation [12], and others. Consequently, it is possible to correlate the rise of ROS in the male reproductive tract with a loss of sperm motility [13], morphology [14], and loss of male fertility capacity in general [15]. These infertile individuals often recur to ART to pursue parenthood. However, the gametes' preparation for these techniques often increases the exposure of gametes and embryos to ROS. For spermatozoa, the removal of seminal plasma, as well as sperm processing techniques (centrifugation, light exposure, temperature variations) cause an increase in ROS production and with that a decrease in the chances of achieving a successful pregnancy [18]. To protect gametes and embryos from excessive ROS, culture media are often supplemented with antioxidants.

In this work, we studied the effects of VC (600 μM) and HYP (100 and 500 μM) supplementation on sperm media, under an exacerbated OS condition. According to the WHO manual for semen analysis [43], after semen collection, samples should be treated as soon as possible, not surpassing the 1-hour time-point, to better preserve spermatozoa physiology [43]. Taking this into consideration, we established the timeframe of 1h to test the cytoprotective effects of VC and HYP media supplementation on spermatozoa. The dosage of VC was chosen based on several studies [40, 42, 51]. The existing literature describes the seminal plasma VC levels to vary between 200-700 μM. We established the concentration of 600 μM of VC to act as the antioxidant control for our study [40, 42, 51]. Considering the lack of literature about the potential effects of HYP on human spermatozoa, a cytotoxicity test was performed with a range of HYP concentrations (0, 5, 50, 100, and 500 μM). These concentrations were chosen considering the existent data regarding the flavonol quercetin, the hyperoside aglycone [49, 52]. Similar to these works, we studied the cytotoxicity effects of HYP to spermatozoa by evaluation of sperm motility and vitality after the treatment. Since we could not detect any alterations regarding sperm motility and vitality after the 1h treatment with increasing doses of HYP, the concentration of 100 and 500 μM were chosen for further testing. This decision was based on the published works regarding the antioxidant effects of quercetin in human spermatozoa [49, 52].

In our study design, we used H_2O_2 as an OS-inducer. However, existing literature regarding the OS-inducer potential of H_2O_2 in *in vitro* studies of spermatozoa is very divergent. Evdokimov, V., and colleagues [53] showed that after 1h of incubation in the presence of 100 μM of H_2O_2, there was an increase in the progressive motility of spermatozoa, while other authors report a decrease of 36% in progressive motility after 1h with 120 μM of H_2O_2 [45]. Otherwise, it was proven that higher concentrations of H_2O_2 (600 μM) cause a drastic reduction in sperm motility and viability after 10 minutes of incubation [44, 45]. In order to promote OS in spermatozoa, but not induce a total immobilization (and death), we selected the concentration of 300 μM of H_2O_2 for our study. According to our results, this concentration of H_2O_2 was able to induce a severe decrease in sperm motility, while no alterations regarding sperm vitality were detected. Thereby the effects observed in sperm motility are not due to cell death but a consequence of the effect of H_2O_2 on motility machinery. As in other studies, sperm motility was chosen as the main indicator of sperm function [49, 52]. Furthermore, sperm motility can be severely affected by OS, probably through inhibition of glucose-6-phosphate dehydrogenase (G6PD) by H_2O_2, leading to a decrease in the availability of NADPH and the consequent peroxidation of membrane lipids [10, 54]. In turn, lipid peroxidation leads to the formation of reactive aldehyde compounds, such as 4-HNE, that are linked to numerous adverse effects on sperm function [55-57].

Our results revealed that the incubation of spermatozoa in media supplemented with VC (600 μM) and HYP (100 and 500 μM) for 1h did not induce deleterious effects in human spermatozoa. The spermatozoa motility after incubation in the antioxidant-only supplemented groups was similar to that of the control group. Also, there were no alterations in the expression of OS biomarkers in these groups. The levels of DNA fragmentation, as well as the MMP, were also similar between the control group and the antioxidant-only supplemented groups. To our knowledge, there is no information regarding the impact of HYP in the physiological and metabolic biomarkers assessed in this work. However, some authors have already studied the impact of VC medium supplementation in spermatozoa [58, 59]. Raad G. and colleagues supplemented sperm swim-up medium with increasing doses of VC (in μM: 0, 300, and 600). The swim-up technique is used for sperm differentiation and isolation, and it is based on the movement of pre-washed spermatozoa. Only spermatozoa with normal physiology can swim up into the overlying medium. Similar to our results, the authors could not find any alteration in sperm-quality parameters (morphology and motility), after the swim-up procedure with and without VC (300 and 600 μM) [58]. Nevertheless, the concentration of 300 μM VC presented lower levels of abnormal chromatin integrity in comparison to the raw semen sample [58]. Our results did not reveal any alteration in the percentage of DNA fragmentation between the control group and the antioxidant-supplemented groups after 1h treatment. However, we must take into consideration that Raad G. and colleagues only considered the spermatozoa from the overlying medium, after the swim-up technique. Abnormal spermatozoa are an endogenous source of ROS, and the fact that we used the total sperm sample could be the reason no statistical differences were found between our control group and the antioxidant-supplemented groups [58]. The authors also reported that the concentration of 600 μM of VC did not affect ROS generation. This finding is in line with results reported by another group, Li and colleagues, which reported that the supplementation of cryopreservation medium with VC (300 μM) significantly reduced the ROS levels in post-thaw spermatozoa [59]. Nevertheless, the concentration of 600 μM VC did not show any difference regarding ROS generation [59]. Li and colleagues justified this result by the fact that VC presents a scavenger activity mainly against·OH and O_2^-, which are important substrates for H_2O_2 formation. The product of this reaction is ascorbyl radical A which induces a series of radical free reactions if accumulated in higher doses [59]. Our results are in accordance with both these studies since we could not find any difference between the expression of OS biomarkers in spermatozoa in the groups treated with antioxidants-only and the control group.

As a haploid cell with a high motility activity, spermatozoa metabolism has been explored in the last decades. Energy production in spermatozoa can occur through two metabolic pathways, namely oxidative phosphorylation (OXPHOS), which occurs in the mitochondria, and glycolysis, which occurs in the head and the flagellum [60]. Regarding those pathways, there are conflicting data about the main source of ATP in human spermatozoa. It is proposed that the ATP produced by OXPHOS contributes to human sperm motility, but it is not enough to sustain high motility [61]. Meanwhile, other researchers showed that an increased mitochondrial respiratory capacity is associated with increased sperm motility, supporting the crucial role played by OXPHOS in human sperm [62, 63]. During glycolysis, glucose enters the cell and is decomposed by a sequence of events originating ATP and pyruvate as by-products. Apart from glycolysis, pyruvate can also be generated by the oxidation of lactate or by the transamination of alanine *via* alanine aminotransferase (ALT). Then, pyruvate can either be oxidized to acetyl coenzyme A, which can enter the tricarboxylic acid (TCA) cycle, to generate ATP *via* OXPHOS, or be converted to lactate *via* lactate dehydrogenase (LDH) in the cytosol. The latter reaction results in the regeneration of cytosolic NAD^+, which is essential for glycolysis [64]. NAD^+ and NADH cannot cross the inner mitochondrial membrane and for this reason are dependent on a system that transfers reducing equivalents to mitochondria, the malate-aspartate shuttle (MAS). In the cytosol, malate dehydrogenase (MDH) reduces oxaloacetate (OAA) to malate, through oxidation of NADH to NAD^+, which then enters in mitochondria. Once in mitochondria, the reverse reaction is performed by MDH, mediating

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the regeneration of NADH [65, 66]. Herein, malate is a metabolite that plays a crucial role in mitochondrial respiration, being part of the TCA cycle and participating in the restoration of mitochondrial NADH levels [67, 68]. We could not find any data regarding the impact of VC and HYP media supplementation on the metabolism of spermatozoa. Therefore, we analysed human spermatozoa metabolism through quantification of metabolites in the sperm preservation media by ^{1}H-NMR. We were able to quantify pyruvate, acetate, lactate, alanine, and malate. Although we could not find any differences between the control and antioxidants-only media regarding pyruvate, lactate, and malate levels, some interesting differences were found regarding the levels of alanine and acetate. The levels of alanine were higher in the media supplemented with HYP (100 and 500 µM), in comparison to the control and those supplemented with VC. No differences between the control group and the media supplemented with VC (600 µM) were found regarding alanine levels. As mentioned, alanine is mostly used as a source of pyruvate, through the process of alanine transamination [64]. Since the pyruvate levels remain the same between the control and the antioxidant-only groups, HYP may induce the formation of alanine to act as a metabolite reservoir. The levels of acetate were higher when VC (600 µM) and HYP (500 µM) were present in the media than in the control, though no difference was found between the control group and the 100 µM HYP supplemented group. Acetate and the related metabolism of acetyl-coenzyme A are known to play several roles in the cell, including lipid biosynthesis and energy production [69]. A previous study conducted by our group showed that Sertoli cells (SCs) produce and excrete large amounts of acetate into the extracellular medium [70]. In addition, a recent study performed in human spermatozoa found considerable amounts of acetate in the sperm medium [71]. Herein, we hypothesized that the higher levels of acetate in media supplemented with VC (600 µM) and HYP (500 µM), which are the groups with higher amounts of antioxidant, can be associated with a higher rate of lipid biosynthesis.

The scenario changed when we added H_2O_2 as an OS-inducer. As mentioned, the incubation of spermatozoa with H_2O_2 severely decreased sperm motility in comparison to the control group and the spermatozoa incubated in media with antioxidants, even though no alterations were found regarding sperm vitality. This group subjected to H_2O_2 also presented the highest levels of sperm lipid peroxidation and the highest levels of DNA fragmentation. Our results also demonstrate that the presence of VC and HYP in the media was able to revert some of the oxidative damage induced by H_2O_2. Although the spermatozoa incubated with the antioxidants in the presence of H_2O_2 did also present a decrease in sperm motility, this decline was not as accentuated as in the one subjected to H_2O_2-only. This pattern can also be observed regarding sperm lipid peroxidation and DNA fragmentation levels. However, the decrease of MMP in spermatozoa incubated with antioxidant plus H_2O_2 was not different from the decrease of MMP observed in the spermatozoa incubated only with H_2O_2. The antioxidant cytoprotective effects of HYP have already been reported for both lung fibroblast cells, and granulosa cells [26, 27]. In sperm cells, Biagi M and colleagues used a *C. sativa* leaf extract (rich in HYP) to study its protective properties against OS [28], though the particular effects of HYP were not directly explored. The authors incubated swim-up selected spermatozoa with several dilutions of *C. sativa* leaf extract in Biggers-Whitten-Whittingham (BWW) buffer (1:100, 1:200, and 1:500). H_2O_2 (100 µM) was used as an OS-inducer. Similar to our results, the incubation of spermatozoa with the *C. sativa* leaf extract did not decrease sperm motility in comparison to the control group. Furthermore, the authors reported a rise in lipid peroxidation levels of spermatozoa incubated with H_2O_2. Nevertheless, when spermatozoa were incubated with the *C. sativa* leaf extract in the presence of H_2O_2, the lipid peroxidation levels remain similar to the control [28]. Our results are in line with this study since we reported that the presence of HYP (100 and 500 µM) showed a tendency to decrease lipid peroxidation levels associated with high OS induced by the presence of H_2O_2. Zribi N. and colleagues supplemented sperm cryopreservation medium with quercetin (50 µM) [72]. Although the process of cryopreservation induced an increase in sperm DNA fragmentation, the quercetin supplementation was able to improve post-thaw sperm parameters and DNA integrity [72]. These quercetin proprieties were also described in several animal sperm

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studies [73-75]. Our results regarding sperm media supplementation with HYP are in line with these quercetin studies. Our results show that the supplementation of sperm medium with HYP (100 μM) was able to prevent some of the deleterious effects of H_2O_2 in sperm, which is represented by an increase in sperm motility, a decrease in DNA fragmentation, and a decreasing trend in lipid peroxidation levels when compared to the spermatozoa under H_2O_2 effect.

Mangoli E. and colleagues reported that the addition of VC (600 μM) in both neat semen and prepared spermatozoa of normozoospermic samples improved the recovery rate of sperm parameters and DNA integrity following vitrification [40]. A similar result was found by Fanaei H. and colleagues, which incubated swim-up spermatozoa with VC (600 μM) [42]. The authors reported decreased DNA fragmentation levels, reduced lipid peroxidation levels, and an improvement in sperm quality parameters (motility and viability) in comparison to spermatozoa in the control group [42]. This scavenger and DNA protective effect of VC addition has already been described by several authors [42, 58, 76]. Our findings are in accordance with these studies since the sperm media supplemented with antioxidants (VC and HYP) were able to preserve sperm motility and decrease DNA fragmentation when compared to the spermatozoa under the effect of H_2O_2. Additionally, the supplementation with these antioxidants also evidenced a tendency to decrease lipid peroxidation levels in oxidative stress conditions.

Nevertheless, our results suggest that VC and HYP cannot reverse the deleterious effects of H_2O_2 regarding sperm metabolism. Interestingly, incubation of spermatozoa with H_2O_2 promoted a decrease of pyruvate levels in the medium, in comparison to those in the control and supplemented with antioxidants. Pyruvate is a pivotal metabolite that takes part in several metabolic pathways. To understand the metabolic pathways that were promoted by H_2O_2, we investigated how the levels of several other metabolites varied. It was previously reported that pyruvate is mainly converted to lactate in human sperm [77, 78]. The decrease in lactate production found in all groups with H_2O_2, even in the presence of VC and HYP, indicates that it is unlikely for spermatozoa to be producing energy by anaerobic respiration. Alanine production also decreased in the presence of H_2O_2, even when media is supplemented with VC and HYP. Since pyruvate consumption levels are higher in media with H_2O_2, spermatozoa might be using alanine as a source of pyruvate through the process of alanine transamination [64]. To further understand mitochondria function from the spermatozoa of these groups, we can correlate the malate levels and the MMP results. As mentioned, all groups incubated with H_2O_2, even in the presence of antioxidants, presented a decrease in the MMP. Along with that, all these groups also presented a decrease in the production of malate. Malate is an important component of mitochondrial metabolism [65, 66]. The lower MMP and malate levels found in the groups of spermatozoa treated in the presence of H_2O_2 suggest a decline in mitochondrial activity, which can also be related to loss of motility in those groups. Therefore, the higher consumption of pyruvate found in these groups appears to not be redirected to the mitochondrial metabolism. Nevertheless, we found that the production of acetate was increased in all H_2O_2-treated groups. The overproduction of acetate is known to be promoted by situations of exacerbated OS [69]. In fact, the reaction of exogenous pyruvate with H_2O_2 is known to induce the formation of acetate, carbon dioxide, and water [79]. In addition, it is also reported that under severe OS situations, pyruvate can exert a protective effect on several cellular systems, acting as a peroxide scavenger [79]. In normal conditions, pyruvate plays a little role in the clearance of H_2O_2 since its concentration and availability are regulated by metabolism. Several authors have explored the antioxidant role of pyruvate in the brain of mice since neurons are highly susceptible to OS [79, 80]. In human cells, Babich H. and colleagues studied the susceptibility of human fibroblast cells to H_2O_2 when treated in culture media with and without pyruvate, where a cytoprotective action of pyruvate was observed [81]. Having this in consideration, we propose that the decrease of pyruvate levels found in the media of spermatozoa treated with H_2O_2 is likely caused by its reaction with H_2O_2, forming acetate, carbon dioxide, and water in the process. Given the important role that pyruvate plays in cellular energy production, we

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2022;56(S1):1-23

DOI: 10.33594/000000487
Published online: 8 January 2022

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hypothesize that the overflow of pyruvate to acetate can disrupt spermatozoa metabolism. Due to the unavailability of pyruvate, other metabolic pathways, such as anaerobic and aerobic respiration are compromised. This would also justify the loss of sperm motility found in the groups treated with H_2O_2, even in the presence of antioxidants. Nevertheless, the loss of motility was attenuated by the antioxidants' presence. Notwithstanding, the levels of acetate found in the group treated with HYP (100 μM) plus H_2O_2 were lower than the levels of acetate found in the spermatozoa treated with 300 μM H_2O_2. This could indicate that supplementation with 100 μM of HYP is more efficient in the protection of spermatozoa towards H_2O_2, most likely through a better neutralization of the available H_2O_2.

Our findings demonstrate that HYP and VC were effective in protecting human spermatozoa against oxidative damage. Nevertheless, the treatment with HYP (100 μM) presented better results regarding sperm preservation than HYP (500 μM) and VC (600 μM). Also, HYP was shown to be more effective than VC (600 μM) since lower concentrations of HYP presented similar protective effects in human spermatozoa. Previous studies reported that VC stability is influenced by pH, temperature, oxygen, and the presence of metal ions, such as copper and iron [82, 83]. VC decomposes rather quickly in an aqueous medium, and its oxidation is highly favoured in neutral or basic conditions, being the lowest rate of oxidation observed at pH 3 [84]. On the other hand, a recent study showed that HYP is stable at pH 4-8, remains unchanged at temperatures between 25-40 °C and presents an excellent photostability [85]. The stability of HYP under physiological conditions (pH 7.4) combined with its high antioxidant capacity enhances the use of this phytochemical in sperm preservation solutions.

Conclusion

For all the aforementioned, we concluded that the supplementation of sperm preservation media with VC and with HYP can be beneficial for the preservation of spermatozoa to ART protocols. These antioxidants appear to present cytoprotective proprieties to human spermatozoa, preserving sperm motility and preventing DNA fragmentation, under oxidative stress conditions. Also, these antioxidants appear to prevent (to some extent) the lipid peroxidation of the spermatozoa membranes from oxidative injury. Our findings evidence that HYP holds a higher antioxidant capacity than VC since the concentration of 100 μM of HYP presented the best results regarding spermatozoa physiology and metabolism. Notwithstanding, the deleterious effects promoted by H_2O_2 found in mitochondrial activity and sperm metabolism could not be reversed by the presence of VC and HYP in the sperm media. To our knowledge, this is the first study to explore the role of HYP as an antioxidant supplement of sperm medium and the first to explore the metabolic consequences of the presence of VC and HYP in sperm preservation medium. Nevertheless, the ideal doses of these antioxidants for the most effective cytoprotective effects in human sperm cells need to be further explored.

Acknowledgements

Cellular Physiology and Biochemistry

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DOI: 10.33594/000000487
Published online: 8 January 2022

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to the discussion of the article. BMS, MGA, and PFO contributed to article conception and design, writing, bibliographic enrichment, data interpretation, and critical discussion. All authors approved the final version for submission.

Statement of Ethics

This study was approved by the Joint Ethics Committee of *Centro Hospitalar Universitário do Porto* (CHUP)/Institute of Biomedical Sciences Abel Salazar CHUP/ICBAS (2021/CE/ P002[P342/CETI/ICBAS]). All procedures were performed in accordance with the local, national, and European ethical guidelines. All patients participated in this study voluntarily. They were informed about the purpose of the study and signed informed written consent.

Disclosure Statement

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Cell Physiol Biochem 2022;56(S1):1-23

DOI: 10.33594/000000487
Published online: 8 January 2022

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