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Influences of the polymorphisms of the *Sod2* gene (rs4880) on the motility and vigor of X- and Y-bearing sperm at different pH values

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ABSTRACT

Superoxide dismutase 2 (SOD2) is an antioxidant enzyme that appears phylogenetically conserved. However, functional *Sod2* polymorphisms have been studied, and the specific polymorphisms are related to activity alterations of the SOD2 enzyme. An example of a polymorphism of SOD2 is Val16Ala (rs4880), which has been identified in exon 2 of the human *Sod2* gene. This polymorphism is recognized as a single nucleotide polymorphism (SNP) and alters the conformation of SOD2. Additionally, recent studies have shown that the Ala16 Val polymorphism in *Sod2* can be related to different pathological diseases. In these terms, the objective of the present study was to evaluate whether the polymorphism of SOD2 in Val16Ala (rs4880) influences the motility and vigor of X- and Y-bearing sperm at different pH values promoting sperm selection. We found that polymorphism rs4880 at normal pH conditions can result in alterations in the activity of superoxide dismutase in the sperm through different assay analyses. Moreover, compelling modulation evidence indicates that this effect could also mediate seminal plasma redox alterations and consequently can play an important role in sperm physiology, fertilization, and postfertilization.

1. Introduction

Sperm metabolism is responsible for maintaining physiological and biochemical functions during the sperm cell life span. Additionally, to respond to the higher energetic demand that sperm cells need, bioenergetics are sustained by innumerous mitochondria on the cell [1]. Mitochondrial metabolism in sperm cells is responsible for producing higher levels of reactive oxygen species (ROS) [2]. During homeostasis, ROS production is considered essential for different cellular signaling pathways. In these terms, the role of ROS production in the sex selection of sperm is yet unclear [3].

Recently, there has been a recognized increase in demand for methods for preconception sex selection [4]. Additionally, technologies that improve the separation of X- and Y-bearing sperm have been described in different studies [5,6]. These advances in methodologies make it possible for parents to select the sex of their future child. In sex selection, ROS can principally influence the maturation and viability of sperm [7]. In these terms, the antioxidant defenses present in sperm cells represent an important way to investigate whether X- and Y-bearing sperm are tolerated or influenced by ROS.

Additionally, given that innumerous events such as influx of calcium and bicarbonate, increases in membrane fluidity, membrane raft redistribution, and pH modulation, which are mediated in sperm cells through ROS signaling pathways, the study of antioxidant properties is also important to investigate the vulnerability of sperm cells to alterations in redox status [8,9]. When evaluating the redox status, it is known that excess ROS induces a cellular condition called oxidative stress. In sperm cells, oxidative stress mediates a series of events, such as damage to DNA, lipoperoxidation, protein oxidation, and alterations in thiol levels in the cells, which culminate in the loss of sperm function [10,11].

Antioxidant enzymes such as catalase (CAT) (EC 1.11.1.6),

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superoxide dismutase 1 and 2 (SOD1; SOD2) (EC 1.15.1.1), and glutathione peroxidase (GPX1) (EC 1.11.1.9) are important mechanisms used by sperm cells to detoxify the exacerbated ROS produced during cellular metabolism [12]. In this way, SOD2, which is localized on the mitochondrial matrix, participates as a key regulator of cellular sperm viability [13]. The key role that SOD2 plays is principally due to a central position in different signaling pathways involved in cellular homeostasis. Moreover, SOD2 is directly involved in the detoxification of superoxide radicals produced in the mitochondria [13]. Recently, functional *Sod2* polymorphisms have been studied, although SOD2 appears to be phylogenetically conserved [14–16]. The specific polymorphisms are related to alterations in the activity of the SOD2 enzyme. An example of a polymorphism of SOD2 is Val16Ala (rs4880), which has been identified in exon 2 of the human *Sod2* gene [17]. This polymorphism is recognized as a single nucleotide polymorphism (SNP) and alters the conformation of SOD2 [18]. Additionally, recent studies have shown that the Ala16 Val polymorphism in *Sod2* can be related to different pathological diseases [15,16,18]. In these terms, the objective of the present study was to evaluate whether the polymorphism of SOD2 in Val16Ala (rs4880) influences the motility and vigor of sperm bearing at different pH values, promoting sperm selection.

2. Material and methods

2.1. Participants

The research design of our study was determined first through the sample number definition using Epi Info™ V.6 software. Samples were obtained from healthy volunteers who consented to participate in the study by signing a consent form. In the first phase of the process, 80 blood samples were collected for DNA extraction and subsequent genotyping, aiming to reach 15 samples for each Ala/Val polymorphism allele of the *Sod2* gene polymorphism, rs4880. The second phase was semen collection for the extraction of sperm targets of the study. In the second phase, only volunteers who had confirmation of the genotypes corresponding to the study were selected to collect the sperm. This study was approved by the Research Ethics Committee and the Ethics Committee for the Use of Animals (Protocol 25325) of the Federal University of Rio Grande do Sul. This study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Preparation of samples and experimental groups

Samples were obtained by masturbation after 3–5 days of intercourse abstinence. After complete liquefaction of the sample, semen was centrifuged, the seminal plasma was carefully aspirated, the cell pellet was washed in 1x DMEM (Dulbecco's modified Eagle's) cell medium, and the samples were concentrated at 35×10^6 spermatozoa/mL. Three aliquots using equal volumes were prepared for the swim-up technique in Quinn's SpermWash Media (HTF) with modified pH: control 7.6 pH, 7.0 pH, and 5.5 pH [19]. All the tests were realized without knowing the genotype of the samples (47CC or 47TT).

2.3. Genotyping

Blood was drawn to the genome, and DNA was extracted from leukocytes by a standard method [20]. Polymerase chain reaction (PCR) was performed in a total volume of 25 μL and contained 10–100 ng of genomic DNA, 1.6 U Taq DNA polymerase in Taq buffer (Life Technologies™, Brazil Ltda. INVITROGEN Inv. São Paulo, SP, Brazil), 0.2 mM of each dNTP, 2 mM $MgCl_2$, and 10% DMSO (dimethyl sulfoxide). The exon 2 segment of the SOD2 gene was amplified using the following primers: sense, 5′-GCC CAG CCT GCG TAG ACG GTC CC-3′ and anti-sense, 5′-TGC CTG GAG CCC AGA TAC CCC AAG-3′ (Life Technologies™, Brazil Ltda. INVITROGEN Inv. São Paulo, SP, Brazil), the underlined nucleotide represents a deliberate primer mismatch designed to introduce an artificial restriction site for analysis of the 47C > T SOD2 SNP. Reactions were subjected to an initial denaturation at 95 °C for 6 min, followed by 35 cycles at 95 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min and 30 s, and a final extension of 7 min in a PTC-100 thermocycler (MJ Research, Inc. Watertown, MA, USA). The PCR amplified product was cleaved in appropriate buffer with 10 U of the *Hae*III restriction endonuclease (GibcoBRL®-Life Technologies™ Rockville, MD, USA) at 37 °C for 8 h. A minimum of 15% of the samples were subjected to a second, independent PCR restriction fragment length-polymorphism analysis to confirm their genotypes.

2.4. Total radical-trapping antioxidant parameter (TRAP assay)

The nonenzymatic antioxidant cellular defenses were estimated by the total radical-trapping antioxidant parameter, which determines the nonenzymatic antioxidant potential of the sample, as previously described [21]. In brief, the reaction was initiated by injecting luminol and 2,2-azobis[2-methylpropionamidine]dihydrochloride (AAPH), a free radical source that produces peroxyl radicals at a constant rate, in glycine buffer (0.1 M, pH 8.6), resulting in a steady luminescent emission. Plasma seminal samples (10 μg of protein) were mixed in glycine buffer in their action vial, and the decrease in luminescence was monitored in a liquid scintillation counter for 60 min after the addition of the samples. The area under the curve obtained of the chemiluminescence values was transformed to percentage values and compared against the control values. The total antioxidant response (TAR) was calculated through the chemiluminescence emitted from samples at 1 min of the experiment.

2.5. Antioxidant enzyme activities

Catalase (CAT) activity was assayed by measuring the rate of decrease of H_2O_2 absorbance in a spectrophotometer at 240 nm [22], and the results are expressed as U/mg protein. Superoxide dismutase enzyme activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline autooxidation in a spectrophotometer at 480 nm, as previously described [23]. The results are expressed as U/mg protein. To determine glutathione peroxidase (GPx) activity, the rate of NAD(P)H oxidation was measured in a spectrophotometer at 340 nm in the presence of reduced glutathione, tert-butyl hydroperoxide, and glutathione reductase, as previously described [24]. A ratio between SOD activity and CAT activity (SOD/CAT ratio) was applied to better understand the effect of the *SOD2* gene polymorphism rs4880 on these two oxidant-detoxifying enzymes that work sequentially by converting the superoxide anion to water. An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress. All the results were normalized by the protein content using the Lowry method [25].

2.6. Motility, acrosome integrity and viability

After Swim-up, a fraction of the sample is collected for its parameters. For motility, the sample was added to a microscope slide for visual inspection analysis by optic microscopy (400x). The motility of spermatozoa was classified into three stages. Briefly, rapid and slow progressive motility was calculated by the speed at which sperm moved with flagellar movement in a given volume as a percentage (range 0–100%) by counting 200 sperm. Then, the motility of spermatozoa was classified into the following three stages: A) rapid progressive motility (> 25 μm/s at 37 °C and > 20 μm/s at 20 °C); B) slow or sluggish progressive motility; and C) nonprogressive motility (< 5 μm/s). For the analysis of acrosome integrity and viability, the double staining technique (trypan blue/Giemsa-TBG) was used [26]. Twenty microliters of trypan blue 0.4% (Sigma Aldrich, Brazil) was stored in a 20 μL microtube. After incubation at 37 °C in a water bath for 15 min, smears were

made, and the blades were dried. Next, the samples were fixed in methanol for 5 min, dried again and immersed in Giemsa solution overnight. A 200 sperm count of each sample was made with a light microscope and oil immersion objective (100x). The sperm were classified into the following three distinct classes: AIA - Spermatozoa Alive with Intact Acrosome: pinkish head with pinkish acrosome; AWA - Spermatozoa Alive Without Acrosome: pinkish or white head with acrosome bleached; D - Spermatozoa Dead: blue head.

2.7. Extraction and quantitation of DNA

DNA was extracted from aspirated spermatozoa of supernatant from the top of the meniscus after swim-up using standard protocol phenol-chloroform extraction [27]. The amount of DNA was determined by a Quantifiler® Duo Kit (Applied Biosystems).

2.8. Real-time PCR amplification

Real-time PCR amplification reactions contained 10.5 μL of Primer-Probe Mix, 12.5 μL of Master Mix, and 2.0 μL of DNA sample. Primer-Probe Mix contained forward and reverse primers and TaqMan® probes for ribonuclease P RNA component H1 (RPPH1), a sex determining region Y (SRY), and internal PCR control (IPC) targets. The IPC template, a synthetic polynucleotide, was cloned into a plasmid. Master Mix contained reference dye, dNTPs, dUTP, $MgCl_2$, AmpliTaq® Gold DNA polymerase and preservatives in Tris-HCl, pH 8.0. Pooled human male genomic DNA at eight different concentrations (50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 μg/μL) was amplified on each quantification run plate to generate standard curves for RPPH1 and SRY targets. Amplification reactions were performed in a 7500 Real-Time PCR System (Applied Biosystems) following the manufacturer's instructions with the following conditions: 50 °C, 2 min; 95 °C, 10 min; 40 cycles of 95 °C, 15 s and 60 °C, 1 min. The data were analyzed using 7500 System Software v1.2.3 (Applied Biosystems) with a threshold value of 0.2.

2.9. Statistical analysis

The results are expressed as the mean ± SEM of at least three independent experiments, and each sample was analyzed in triplicate. Data were analyzed by Student's *t*-test. When appropriate, one-way analysis of variance (ANOVA) and individual group means were compared using Tukey's multiple group comparison test. Differences were significant when $P < 0.05$. Data analyses were performed with SPSS 17.0 (SPSS, Inc.).

3. Results

We first evaluated the polymorphism of *Sod2* in Val16Ala (rs4880) on sperm motility at different pH values. Our results showed that sperm that present the 47 T allele in the Sod2 gene at pH 5.5 and pH 7.0 and under control conditions present higher motility (Fig. 1A). In terms of viability and acrosome integrity, we found that at pH 5.5 and pH 7.0, a significant decrease in sperm viability was observed, independent of the allele in the *Sod2* gene (Fig. 1B). Moreover, the percentage of live spermatozoa did not present a difference in terms of the allele in the *Sod2* gene at either pH (Fig. 1B). Interestingly, under the control conditions, we found that sperm that present the 47 T allele in the *Sod2* gene present a decrease in viability. The percentage of live spermatozoa with intact acrosomes was also lower in sperm that presented the 47 T allele in *Sod2* (Fig. 1B). DNA analysis also revealed that under control conditions, the 47 T allele in the *Sod2* gene had a higher presence in male DNA and the 47 C allele had a higher presence in female DNA. Surprisingly, at pH 7.0, the presence of the 47 T allele in the *Sod2* gene was higher in female DNA (Fig. 1C). At pH 5.0, we did not observe differences in the content of male and female DNA.

After the characterization of sperm mobility, viability, and DNA sex evaluation, we performed redox assays in the sperm to determine the oxidative parameters. We investigated the activity of antioxidant enzymes. In terms of glutathione peroxidase (GPx), independent of pH conditions, GPx activity did not change (Fig. 1D). Moreover, our results reveal that GPx activity is not altered in the different samples that exhibit polymorphism of *Sod2* in Val16Ala (rs4880). SOD activity was also evaluated, and our results revealed that under control conditions, there was a difference in SOD activity related to the polymorphism of *Sod2* in Val16Ala (rs4880) (Fig. 1E). The presence of the 47 T allele in the *Sod2* gene was correlated with a decrease in SOD activity in the control group. Interestingly, at pH 5.0 and pH 7.0, we did not observe differences in SOD activity, independent of the polymorphism of *Sod2*. Although our results revealed alterations in SOD activity, these modifications did not result in an imbalance in the SOD/GPx ratio in the samples (Fig. 1F). In the total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR) evaluated in the different conditions in which the sperm were submitted, we did not observe differences in our results (Fig. 1G and H, respectively). Therefore, these results reveal that in terms of nonenzymatic antioxidant defenses, the polymorphism of *Sod2* and pH conditions did not alter these parameters in the sperm. Taken together, our data reveal that polymorphisms can modulate SOD activity; however, this did not alter the nonenzymatic antioxidant defenses in the sperm.

Seminal plasma was also evaluated in the different polymorphisms of *Sod2*. Our results reveal that the seminal plasma of individuals with the present 47 T allele in *the Sod2* gene presented an increased activity of CAT compared with the seminal plasma of individuals with the present 47 C allele in the Sod2 gene (Fig. 2B). We also observed that TRAP in seminal plasma was significantly different between individuals with polymorphisms of *Sod2*. Individuals who presented the 47 C allele in the *Sod2* gene showed a higher level of TRAP than individuals who presented the 47 T allele in the *Sod2* gene (Fig. 2E). TAR did not differ between individuals with polymorphisms of *Sod2*. Additionally, we investigated the genotype frequencies versus gender of the population.

4. Discussion

Sperm sex selection has emerged as a field of reproductive methods. Different researchers have conducted studies to optimize sperm selection and have consequently used assisted reproductive technology experiments [28,29]. Additionally, sperm metabolism is also considered a key factor in terms of the cell viability during the physiological process of reproduction. The redox homeostasis of sperm is regulated through different mechanisms, which involve cellular signaling pathways and antioxidant defenses [30]. Alteration of metabolism can consequently modulate the redox status of the sperm and culminate in functional damage [31]. Here, we evaluated whether functional *Sod2* polymorphisms can alter the physiological and redox parameters of sperm under different pH conditions.

Alterations in the redox status of sperm are principally related to male infertility. DNA damage, lipid peroxidation, and mitochondrial alterations are effects observed in sperm, principally associated with higher levels of ROS production [32]. Specifically, in sperm, mitochondria are retained in the cell, while a significant amount of cytoplasm is removed during the process of spermatogenesis [33]. In particular, mitochondria are responsible for the bioenergetics of sperm. Moreover, mitochondria are considered one of the main sources of cellular ROS production [34]. In sperm, due to the high number of mitochondria in the cell, the cellular content is more exposed to alterations mediated through mitochondrial metabolism. In these terms, mitochondrial ROS production is principally related to elevated levels of superoxide (O_2•-) production, and complex III of the electron transport chain has been recognized as a mitochondrial source of O_2•- production [35,36]. To counteract O_2•- production, the antioxidant enzyme SOD2, which is widely known as MnSOD (manganese SOD) and is localized on

the mitochondrial matrix, mediates the conversion of $O_2\bullet$- to H_2O_2 at a 2:1 ratio [37]. Therefore, the importance of SOD2 activity in this way can be directly related to homeostasis, quality and viability of the sperm.

In terms of motility, it is known that exacerbated levels of ROS, such as $O_2\bullet$-, can directly induce the oxidation of polyunsaturated fatty acids (PUFAs) in sperm cells [38]. The sperm membranes are enriched with PUFAs, and any alterations in composition or structure can directly influence male fertility [39]. The high content of PUFAs contributes directly to membrane fluidity and flexibility. Additionally, the metabolites generated through PUFA oxidation, which are known as secondary products of lipoperoxidation, can mediate alterations in cellular or tissue signaling pathways [40]. Therefore, SOD2 plays a critical role in the sperm environment, principally because SOD2 mediates the protection of both mitochondria and cellular content against the exacerbated

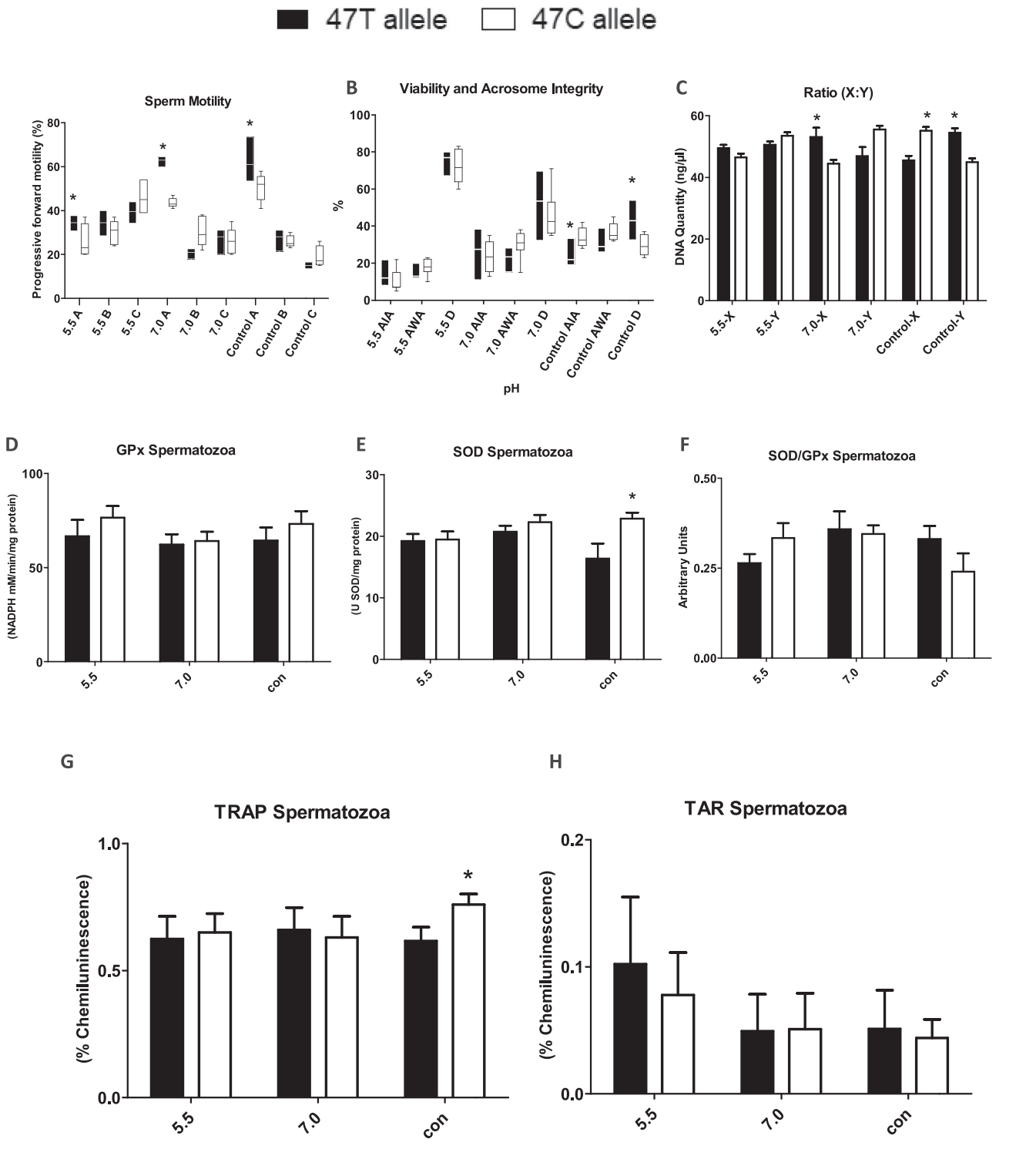

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Fig. 1. *Sod2* polymorphism influence the motility, viability, acrosome integrity, ratio of X and Y chromosome and cellular redox environmental with decreasing pH, all techniques were performed after the swim-up. (**A**) Motility is the percentage of motile sperm among the total of 200 cells, the motility of spermatozoa was classified in three stages: A) rapid progressive motility (> 25 μm/s at 37 °C and > 20 μm/s at 20 °C); B) slow or sluggish progressive motility; C) nonprogressive motility (< 5 μm/s). Student's *t*-test was performed; **P* < 0.05 value describes a comparison between 47 C and 47 T allele; mean of three replicates (n = 30) and the values are expressed as the mean ± SEM. (**B**) Viability and Acrosome Integrity - Alive with Intact Acrosome (AIA), Alive Without Acrosome (AWA), and Dead (D). Student's *t*-test was performed; **P* < 0.05 value describes a comparison between 47 C and 47 T allele; mean of three replicates (n = 30) and the values are expressed as the mean ± SEM. (**C**) Representative graphs showing the ratio of female DNA and male DNA extracted from the cells after swim-up. Student's *t*-test was performed; **P* < 0.05 value describes a comparison between 47 C and 47 T allele; mean of three replicates (n = 30) and the values are expressed as the mean ± SEM. (**D**) GPx activity the rate of NAD(P)H oxidation was measured in a spectrophotometer at 340 nm in the presence of reduced glutathione. Student's *t*-test was performed; mean of three replicates (n = 30) and the values are expressed as the mean ± SEM. (**E**) Total SOD activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm. Student's *t*-test was performed; **P* < 0.05 value describes a comparison between 47 C and 47 T allele; mean of three replicates (n = 30) and the values are expressed as the mean ± SEM. (**F**) A ratio between SOD activity and GPx activity. Student's *t*-test was performed; mean of three replicates (n = 30) and the values are expressed as the mean ± SEM. (**G**) TRAP index was measured by luminol-enhanced chemiluminescence. Student's *t*-test was performed; **P* < 0.05 value describes a comparison between 47 C and 47 T allele; mean of three replicates (n = 30) and the values are expressed as the mean ± SEM. (**H**) TAR was determined through the chemiluminescence emitted from samples at 1 min of experiment. Student's *t*-test was performed. Mean of three replicates (n = 30) and the values are expressed as the mean ± SEM.

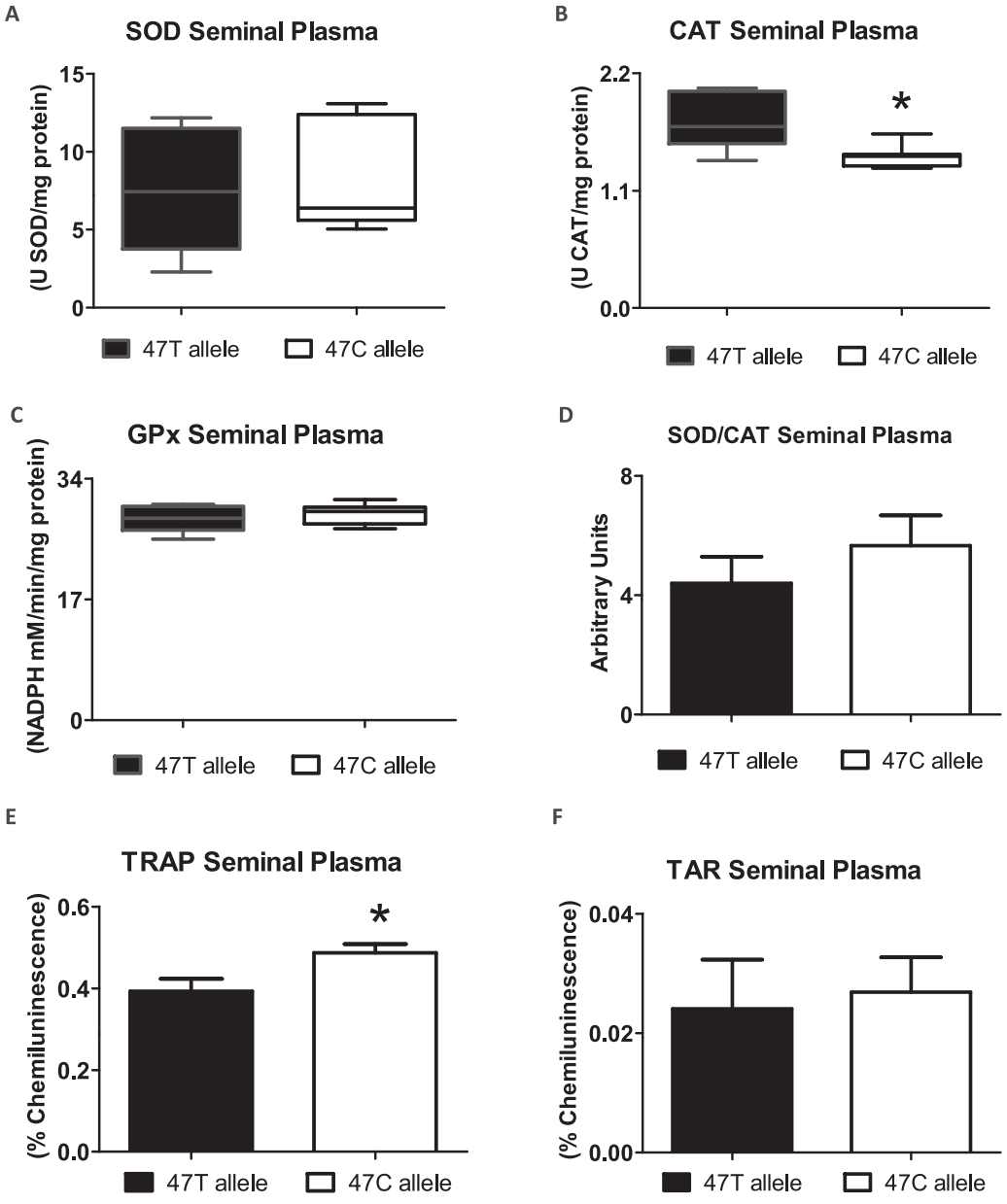

Fig. 2. *Sod2* polymorphism influence redox environmental in the seminal plasma. (**A**) Total SOD activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm. Student's *t*-test was performed. Mean of three replicates (n = 30) and the values are expressed as the mean-± SEM. (**B**) Catalase activity was assayed by measuring the rate of decrease in H2O2 absorbance in a spectrophotometer at 240 nm. Student's *t*-test was performed; **P* < 0.05 value describes a comparison between 47 C and 47 T allele; mean of three replicates (n = 30) and the values are expressed as the mean ± SEM (**C**) GPx activity the rate of NAD(P)H oxidation was measured in a spectrophotometer at 340 nm in the presence of reduced glutathione. Student's *t*-test was performed. Mean of three replicates (n = 30) and the values are expressed as the mean ± SEM. (**D**) Ratio between SOD activity and CAT activity. Student's *t*-test was performed. Mean of three replicates (n = 30) and the values are expressed as the mean ± SEM. (**E**) TRAP index was measured by luminol-enhanced chemiluminescence. Student's *t*-test was performed; **P* < 0.05 value describes a comparison between 47 C and 47 T allele; mean of three replicates (n = 30) and the values are expressed as the mean ± SEM. (**F**) TAR was determined through the chemiluminescence emitted from samples at 1 min of experiment. Student's *t*-test was performed. Mean of three replicates (n = 30) and the values are expressed as the mean ± SEM.

content of O_2•- that is produced during metabolism [41].

The importance of SOD2 is not restricted to antioxidant activity. Recently, it has been suggested that SOD2, through H_2O_2 generation, can promote redox signaling and extend the regulatory action in different cellular compartments [42,43]. Moreover, polymorphism of *Sod2* gene rs4880 has been related to alterations in SOD2 structure [44]. Conformational changes in polymorphism rs4880 can affect the efficiency of the transport of SOD2 into mitochondria [45]. Additionally, this polymorphism of the *Sod2* gene has also been associated with susceptibility to developing proinflammatory states and an increased risk for metabolic alterations such as higher levels of cholesterol and glucose and modulation of leptin levels [46]. In terms of sperm, different studies have evaluated the possible association of the *Sod2* gene polymorphism rs4880 with alterations in the motility, quality and viability of the sperm [47–49]. Here, our results reveal that sperm under control pH conditions present a significant difference in SOD2 activity when samples were grouped in accordance with the *Sod2* gene polymorphism rs4880. Moreover, our approach also revealed a modulation of redox parameters in seminal plasma, which showed an association with the *Sod2* gene polymorphism rs4880. The role of seminal plasma in sperm fertilization and postfertilization has motivated a growing number of studies [50]. Redox alterations in the seminal plasma can modulate cell signaling and consequently affect the fertilization process, which is a complex physiological process. The relation of the *Sod2* gene polymorphism rs4880 is an innovative aspect to investigate alterations in seminal plasma [47–49]. Additionally, the study of these relationships corroborates the modulation of the redox environment of sperm and fertilization.

In conclusion, to our knowledge, this is the first report that investigated the *Sod2* gene polymorphism rs4880 and the influences of the presence of different alleles on the motility and vigor of sperm bearing at different pH values to promote sperm selection. Our results indicate that polymorphism rs4880 under normal pH conditions can result in alterations in SOD activity in sperm. Moreover, the modulation observed in SOD activity in the sperm provided compelling evidence that this effect could also mediate seminal plasma redox alterations. Therefore, taken together, our results reveal that the *Sod2* gene polymorphism rs4880 influences the redox status of sperm and seminal plasma. In terms of sperm, our findings reinforce the need for further studies to improve the comprehension of the *Sod2* gene polymorphism rs4880 and its involvement in sperm physiology, fertilization, and postfertilization.

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CRediT authorship contribution statement

Conceptualization and Methodology: F.J.O.P, M.A.B.P., and J.C.F.M.; Formal analysis, Writing – original draft preparation, F.J.O.P., M.A.B.P., A.R.V., I.B.O, 1L.V.B.G., D.P.G., and J.C.F.M. All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

The authors do not have any conflict of interest.

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