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In vitro effect of autologous platelet-rich plasma on H_2O_2-induced oxidative stress in human spermatozoa

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ABSTRACT

Background: Among new therapies emerging in the medical field, the use of platelet-rich plasma (PRP) in human reproduction has not yet been explored.

Objectives: This study aimed at investigating the effect of autologous PRP on sperm parameters in the presence and absence of H_2O_2.

Materials and methods: Semen samples were collected from 30 healthy men in their fourth decade attending Azoury IVF clinic. Spermatozoa, cultured in the presence or absence of 10 μM H_2O_2, were left untreated or treated with increasing concentrations of PRP (2, 5, and 10%). After 24-h incubation, ROS levels were assessed and sperm parameters were evaluated.

Results: Our results highlight the harmful effect of H_2O_2 on sperm parameters, showing an increase in the percentage of reactive oxygen species (ROS)-positive cells, vacuolization, and sperm DNA fragmentation, and a decrease in progressive and total motility in the H_2O_2-treated group compared to non-stressed spermatozoa. When samples were treated with PRP, an improvement of the studied parameters was noted mainly with 2% PRP, thus regarded as the best concentration to achieve a positive effect on sperm parameters. Indeed, non-stressed and stressed spermatozoa treated with 2% PRP showed a significant increase in progressive and total motility, coupled with a decrease in ROS-positive cells, DNA fragmentation, vacuolization, and dead cells compared to the untreated group. In contrast, no significant difference in cell morphology was found between the two groups. Moreover, 2% PRP treatment enhanced sperm parameters and prevented cell death in H_2O_2-exposed spermatozoa as compared to freshly collected semen.

Discussion: We suggest that PRP because of its wide arrays of growth factors included in his alpha granules contributes to the inhibition of ROS through the antioxidant, anti-apoptotic activity.

Conclusion: Autologous PRP improves the quality of the sperm, more so in the presence of an H_2O_2-induced OS.

INTRODUCTION

Around 8 to 15% of couples are unable to conceive after one year of unprotected regular intercourse (Stephen & Chandra, 2006). Male factor is exclusively responsible for about 20 to 30% of these cases and contributes, along with a female factor, in another 50% (Agarwal *et al.*, 2015). In addition to the standard semen analysis performed routinely in the laboratory, various specialized tests, such as oxidative stress (OS) measurement, sperm DNA fragmentation, and sperm-head vacuolization, can be realized as part of the etiologic diagnosis of infertility (Benchaib *et al.*, 2007; Sigman *et al.*, 2009). The assessment of these parameters seems to be a predictor of *in vitro* fertilization (IVF) success, embryo quality, and miscarriage rates (Agarwal *et al.*, 2014; Boitrelle *et al.*, 2011; Cho & Agarwal, 2018).

Several studies have shown that OS should be regarded as an important factor of male infertility, thus resulting in poor fertilization, poor embryonic development, or pregnancy loss (Agarwal *et al.*, 2014). OS is defined as an imbalance between the systemic manifestation of reactive oxygen species (ROS) and body's antioxidant capacity. Physiologically, ROS levels are low and controlled by antioxidant systems; their presence being important for many processes such as capacitation, hyperactivation, acrosome reaction, and spermatozoa–oocyte fusion (Agarwal *et al.*, 2014). However, at high concentrations, ROS can

cause sperm DNA damage and may alter the structural configuration of the plasma membrane by affecting its lipids and proteins (Sanocka & Kurpisz, 2004; Aprioku, 2013).

In this regard, multiple antioxidants have been proposed to decrease the effect of OS on spermatogenesis, and therefore as a treatment to improve sperm parameters and maximize the pregnancy rate of a couple. These antioxidants taken orally are being used for at least three months of duration to show a positive impact on sperm quality (Lombardo *et al.*, 2011); thus the importance of an alternative treatment that is fast, effective, and cost-efficient in assisted reproductive technologies, and which objective is to decrease OS and therefore enhance sperm function and quality.

Platelet-rich plasma known as PRP is a novel therapeutic option that is being used in multiple medical fields and has proven promising results in dermatology, orthopedics, and dentistry (Lubkowska *et al.*, 2012). PRP is defined as an autologous concentration of human platelets that is 3 to 5 times greater than physiologic concentration of thrombocytes in whole blood (Knezevic *et al.*, 2016). Its potential therapeutic effect is due to the presence of various factors such as transforming growth factor β (TGF-β), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), zinc (Zn), and superoxide dismutase (SOD) (Appel *et al.*, 2002; Laskaj *et al.*, 2009; Castillo *et al.*, 2011; Magalon *et al.*, 2014). Many of these factors were studied solely on the spermatozoa showing significant positive effect on its quality and function. For instance, TGF-β (Sharkey *et al.*, 2016), FGF (Saucedo *et al.*, 2015), VEGF (Iyibozkurt *et al.*, 2009), Zn (Zhao *et al.*, 2016), and SOD (Perumal, 2014) were found to enhance sperm motility, while IGF-1 (Lee *et al.*, 2016) and PDGF (Basciani *et al.*, 2002) were associated with normal sperm parameters.

Due to its high efficacy and safety, PRP should be considered as a promising therapy that warrants further investigation on its biological effects on sperm quality, and subsequently on the rate of IVF success. Therefore, the objectives of this study were to evaluate sperm OS induced by hydrogen peroxide (H_2O_2) through measurement of ROS levels in sperm cells and to determine the effect of autologous PRP on human sperm parameters affected by H_2O_2 treatment.

MATERIALS AND METHODS

Ethics statement

The study was conducted according to the Declaration of Helsinki and approved by the Ethical Committee of Saint-Joseph University and Hotel-Dieu de France Hospital, Beirut, Lebanon (Ref: HDF 917). Samples (blood and spermatozoa) were collected after obtaining a written informed consent from all participants.

Study population

A total of 30 healthy men attending Azoury IVF Clinic (fertility center) at Mount Lebanon Hospital were assessed in this study. All participants had a normal semen analysis based on sperm parameter evaluation. In-person interviews were conducted to complete a questionnaire about age, current or previous disease status including urogenital ones, and habits such as smoking and alcohol intake. The mean age of participants was 35 ± 5 years.

Semen samples, produced by masturbation and collected into sterile plastic containers, were incubated at 37 °C for 30 min to allow liquefaction. Each sample was then evaluated for sperm concentration, motility, morphology, viability, DNA fragmentation, and ROS levels. The conventional semen profile for all samples was constructed according to the World Health Organization manual for the examination of human semen (WHO 2010).

Participants with a sperm concentration less than 14×10^6 cells/mL were not included in the study. All patients presenting defects in sperm parameters, suffering from andrological disorders, inflammatory and immune diseases, or recent fever, or undertaking any treatment that may alter spermatogenesis were excluded from the study.

Preparation of semen samples

Sperm samples were prepared using density gradient technique according to manufacturer's protocol (Sperm Gradient kit, COOK medical, Australia). Briefly, density gradient media was prepared by layering the 40% medium over the 80% medium, and two milliliters of the liquefied semen sample was added over the upper layer. After centrifugation (380 ***g*** for 10 min), most of the supernatant was gently removed and the pellet was resuspended in two milliliters of sperm medium (Sperm Medium, COOK medical, Australia) to remove the density gradient medium. At the end of the centrifugation (670 ***g*** for 5 min), the final pellet was resuspended in the sperm medium.

Treatment of samples with hydrogen peroxide (H_2O_2)

The resulting pellets ($n = 5$) were cultured in duplicate in the presence of several concentrations of H_2O_2 ranging between 0 and 100 μM with different incubation time. Upon establishment of a dose–response curve for the effect of H_2O_2 on sperm parameters, the suitable concentration and incubation period for the induction of sperm OS were determined.

Blood collection and analysis

Following the optimization of the H_2O_2 concentration, 30 new participants were recruited for the study. Blood was collected from all participants in tubes containing isocitrate anticoagulant, and platelet concentration was evaluated within 30 min using an automatic hematology analyzer (XN-2000, Sysmex, Kobe, Japan). Platelet concentration lower than 150.000/μL and use of any medications that may affect platelet function or concentration for 21 days prior to blood donation were considered as exclusion criteria.

Autologous PRP preparation

The PRP preparation procedure consisted of two centrifugation steps. The initial centrifugation at 380 ***g*** for 15 min at room temperature separates the whole blood into three layers: an upper layer containing mostly platelets and white blood cells (WBC), an intermediate thin layer known as the buffy coat rich in WBC, and a bottom layer consisting mostly of red blood cells (RBC). Most of the RBCs were eliminated, and the upper layer and buffy coat were transferred to an empty sterile tube and centrifuged at 1300 ***g*** for 8 min for PRP collection. A filtration process was then performed to remove WBC (using leukocyte filtration filters), and a PRP platelet count of 1 million/μL has

been considered as the ideal therapeutic dose of PRP (Marx, 2004).

Sperm treatment

To determine the effect of PRP on sperm parameters, cells, control and stressed with 10 μM H_2O_2, were co-cultured in duplicate with 150 μL of sperm medium in 96-well plates in the presence or absence of increasing concentrations of PRP (2, 5, and 10%). After 24-h incubation at 37 °C, sperm parameters were assessed.

Measurement of intracellular reactive oxygen species (ROS)

Sperm H_2O_2-induced OS was evaluated through measurement of ROS in sperm cells. ROS status was assessed using nitroblue tetrazolium (NBT) test as described previously (Esfandiari *et al.*, 2003). Briefly, NBT staining was done by mixing equal volumes of 0.1% of NBT solution and sample (10 μL) and incubation for 30 min at 37 °C. A smear was prepared from the mix, then let to dry, and finally counterstained with Wright stain solution (Sigma-Aldrich, Japan). A total of 200 sperm was evaluated under 100× oil immersion with a bright field microscope. NBT, an electron acceptor, reacts with cellular superoxide ions to form a blue-black compound formazan. ROS-positive spermatozoa were scored as NBT+ when solid deposits of formazan were found in cytoplasm and/or head (Fig. 1).

Sperm motility

Sperm motility was determined under light microscopy using 40× magnification by counting sperm cells in 6 microscopic fields to determine the average percentage and noted as progressive, non-progressive, and immotile, according to WHO 2010 criteria (WHO 2010). Each sample was evaluated by two technicians, in duplicate, and readings were averaged between the two evaluations after calculation of error.

Sperm DNA fragmentation

Halosperm kit (Halotech DNA, Spain) was used as an improved sperm chromatin dispersion (SCD) test to assess sperm DNA fragmentation (SDF) according to manufacturer's instructions. A minimum of 300 spermatozoa per sample were scored under microscope, using the objective 100. Spermatozoa with large or medium-sized halos, characteristic of dispersed DNA loops, were considered to have intact DNA. Spermatozoa with small, degraded, or absent halos were considered to have fragmented DNA.

Sperm morphology and vacuolization

Sperm morphology and vacuolization were evaluated using the Spermoscan Kit (RAL diagnostics, Australia) according to manufacturer's instruction. Briefly, the smears were fixed in the spermo FIX-RAL solution. The sperm cytoplasm and nucleus were then stained using the spermo EOSIN-RAL and the spermo BLUE-RAL, respectively. After drying, a minimum of 200 spermatozoa were counted and assessed for morphology and vacuolization using the optic microscope according to Kruger's strict criteria: head defects: large or small, tapered, amorphous, vacuolated (more than two vacuoles or > 20% of the head occupied by unstained vacuolar areas), small, or large acrosomal areas; mid-piece defects: bent, cytoplasmic residues; and tail defects: coiled, multiple (WHO 2010).

Sperm viability

Sperm viability was evaluated using eosin-nigrosin staining. Ten microliters of each sample was mixed with equal volume of eosin-nigrosin solution. After 30 sec, the samples were smeared and let to air-dry. At least 200 spermatozoa were evaluated under 100× oil immersion with a bright field microscope. Unstained spermatozoa were classified as alive, whereas dead ones with

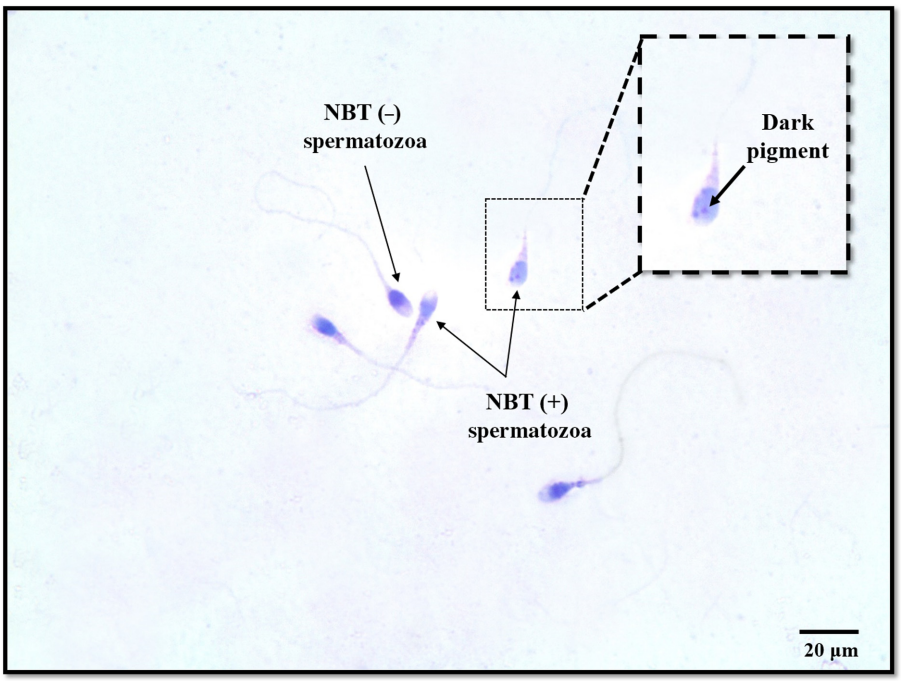


Figure 1 Figure showing the nitroblue tetrazolium (NBT) test in human spermatozoa. Spermatozoa were incubated with NBT, fixed, and stained with Wright stain. NBT molecule interacts with intracellular reactive oxygen species (ROS) to form a dark blue pigment called formazan. Scale bar represents 20 μm. Magnification ×100.

altered membranes were stained pink or red (eosin) with a dark background (high concentration of nigrosin) (WHO 2010).

Observation of spermatozoa by a high-magnification microscope

Sperm samples were recorded at 200× magnification using an inverted microscope (Olympus IX71, Japan) equipped with a high-resolution (1280 × 1024p/15fps) video system (Octax by Vitrolife, Sweden) and an imaging software (Octax EyeWare software, Sweden). Each capture was recorded for 1 min, taken at 0 and 24 h, for the control spermatozoa, and the treated one with 2% PRP.

Statistical analysis

Means and standard deviations were calculated for all variables using the GraphPad Prism software version 6 (GraphPad Software, Inc., USA). The analysis of variance (ANOVA) for non-parametric data was used to assess the variables between groups, followed by Tukey's post hoc test. p values <0.05 were considered statistically significant.

RESULTS

Effect of H_2O_2-induced oxidative stress on human sperm parameters

OS was induced in normal sperm samples using different concentrations of H_2O_2, creating hence an *in vitro* model of stressed specimens mimicking the abnormal spermatozoa of male patients undergoing fertility treatment. A kinetic dose–response study was firstly performed to determine the concentrations of H_2O_2 capable of inducing oxidative stress in sperm cells without causing a detrimental damage. Cells incubated with concentrations higher or equal to 50 μM were found in majority dead after 1-h incubation time, thus opting the 0–40 μM range to evaluate ROS levels and human sperm parameters in the following experiments.

The assessment of OS markers was based on the percentages of ROS-positive cells that were significantly higher in spermatozoa treated for 1 or 2 h with 10, 20, or 40 μM H_2O_2 as compared to the 0 μM H_2O_2 group assessed at the same time-points ($p < 0.0001$). Moreover, all H_2O_2 concentrations induced significant higher ROS levels in sperm cells in comparison with fresh samples directly analyzed after collection (0 h) (Fig. 2A).

The harmful effect of H_2O_2 on sperm parameters was manifested by a decrease in the percentage of progressive and total motility in the treated group as compared to the 0 μM H_2O_2 non-treated spermatozoa (Fig. 2B,D), accompanied with an increase in the percentage of sperm DNA fragmentation (Fig. 2E), vacuolization (Fig. 2F), and dead sperm (Fig. 2G). In contrast, sperm morphology was similar between the two groups (Fig. 2H), keeping a homogenous appearance of spermatozoa autonomously from the incubating medium. While all H_2O_2 concentrations had a negative effect on sperm parameters, the most significant results were noted for the 40 μM concentration where the progressive and total motility were less than the normal values defined by WHO 2010 guidelines (32% and 40%, respectively), and the number of dead cells higher than 50% after 1-h incubation time. The selection of the 10 μM H_2O_2 as the best concentration to induce a notable effect on sperm quality was based on the fact that the 20 μM concentration induced in 1 h a significant decrease in sperm vitality as compared to freshly collected spermatozoa, hence reducing the chance of studying the effect of PRP at a later stage on these samples.

Effect of autologous PRP on human sperm parameters

Determination of the optimal PRP concentration

In order to assess the biological rationale for the use of autologous PRP in male infertility, we first determined the optimal concentration of PRP to improve human sperm parameters. In this regard, stressed and non-stressed spermatozoa were treated with different concentrations of PRP (2, 5, and 10%) for 24 h. Results obtained were then compared with the untreated spermatozoa (0% PRP concentration at 24 h) considered as control and with initial sperm parameters recorded at T0 which corresponds to spermatozoa immediately tested after collection.

The percentage of progressive and total motile spermatozoa was significantly higher in non-stressed spermatozoa treated with 2% PRP compared to the untreated spermatozoa (0% PRP) ($p < 0.0001$ and $p < 0.001$, respectively) (Fig. 3A,C). However, no difference was noted when PRP concentration was increased to 5%, and even the progressive motility was negatively affected when the concentration was further enhanced to 10% showing in the latter group decreased percentages of progressive and total motile spermatozoa compared to controls (Fig. 3A,C). On the other hand, in the stressed group, progressive and total motility significantly improved compared to controls when treated with PRP at concentrations of 2% ($p < 0.0001$) and 5% ($p < 0.001$), but not at a concentration of 10%, where sperm motility was significantly diminished (Fig. 3A,C). Interestingly, the non-stressed and stressed spermatozoa treated with 2% PRP showed significant better progressive and total motility as compared to the 5% PRP-treated group.

All PRP concentrations were able to significantly reduce the percentage of ROS-positive cells (Fig. 3D) and DNA fragmentation (Fig. 3E) in stressed and non-stressed spermatozoa. While similar results were obtained with the 2 and 5% PRP with regard to sperm DNA fragmentation, stressed spermatozoa treated with 2% PRP exhibited significant decreased ROS levels than those treated with 5% PRP ($p < 0.05$).

Regarding sperm vacuolization, the percentage was significantly lower in non-stressed spermatozoa treated with 2 and 5% PRP compared to the control group ($p < 0.0001$ and $p < 0.05$, respectively). In contrast, no difference was noted when samples were treated with 10% PRP. As for the stressed sperm group, PRP treatment significantly improved the percentage of vacuolization, irrespectively of the concentration applied ($p < 0.0001$) (Fig. 3F). Moreover, both non-stressed and stressed spermatozoa showed a better vacuolization rate when treated with 2% PRP as compared to 5% PRP ($p < 0.05$).

Regarding the percentage of dead spermatozoa in stressed and non-stressed specimens treated with 2% PRP, it was significantly lower when compared to the control group ($p < 0.0001$ and $p < 0.001$, respectively). However, no difference was noted when the concentration was increased to 5%, and even the sperm viability was negatively affected when the concentration was further increased to 10% showing in the latter a higher percentage of dead spermatozoa compared to the control ($p < 0.001$) (Fig. 3G). These results clearly reveal that PRP at 2% is the best to preserve sperm viability.

Figure 2 Effect of H_2O_2 on ROS levels (A) and human sperm motility (B-D), DNA fragmentation (E), vacuolization (F), viability (G), and morphology (H). Sperm samples (n = 5) were treated with different concentrations of H_2O_2 ranging between 0 and 40 μM and for different incubation periods. Differences among groups were evaluated using the non-parametric two-way ANOVA test further evaluated by Tukey test for post hoc pairwise comparisons. Data are represented as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ ****$p < 0.0001$ for significance between sperm treated with 10, 20, or 40 μM H_2O_2 and the non-treated group (0 μM H_2O_2) at a same time-point. ˙$p < 0.05$; ¨$p < 0.01$; ˙˙˙$p < 0.001$ ¨¨¨$p < 0.0001$ for differences between spermatozoa exposed to the same H_2O_2 concentration for different incubation periods and sperm immediately analyzed after collection (0 h of incubation time).

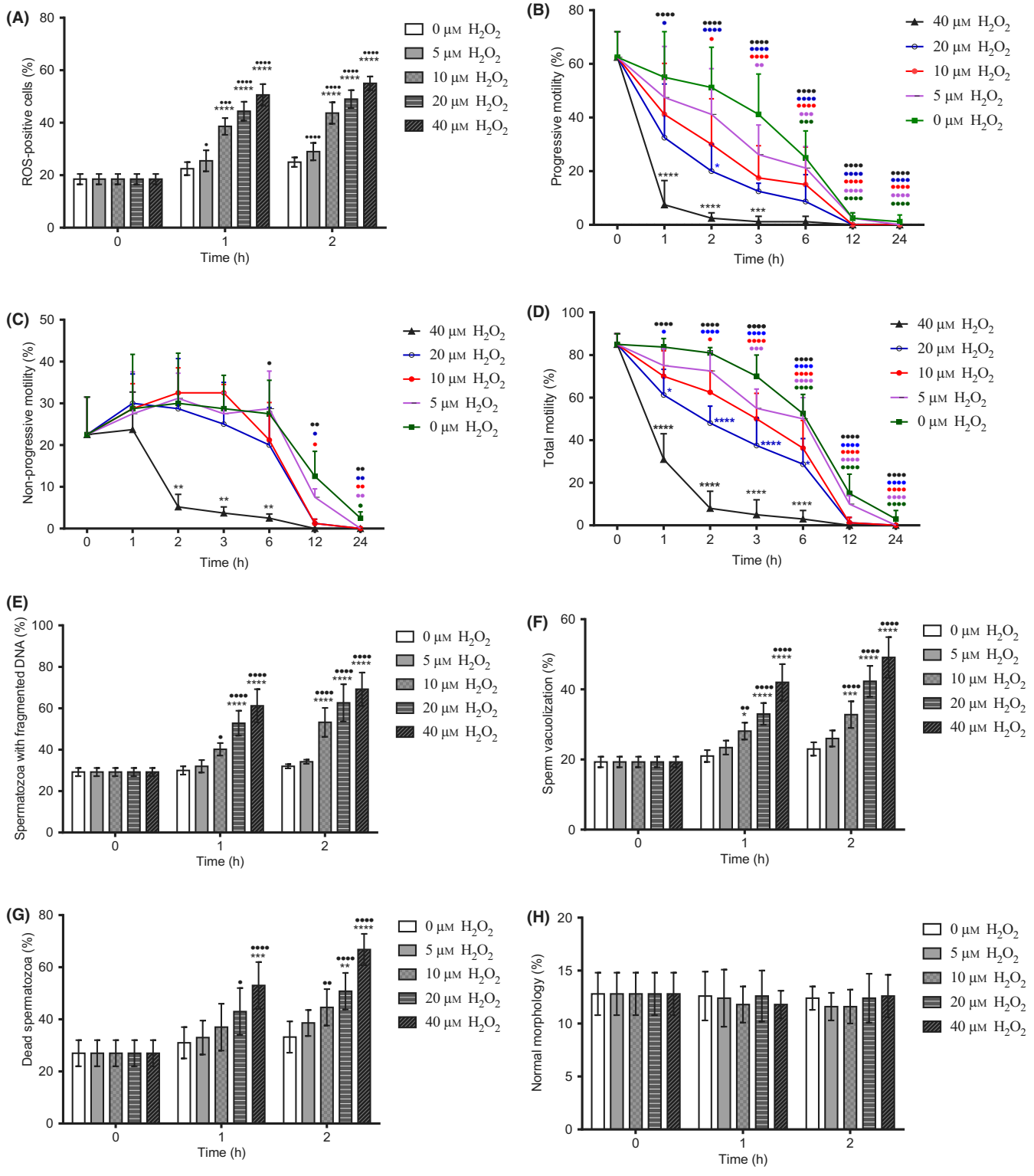
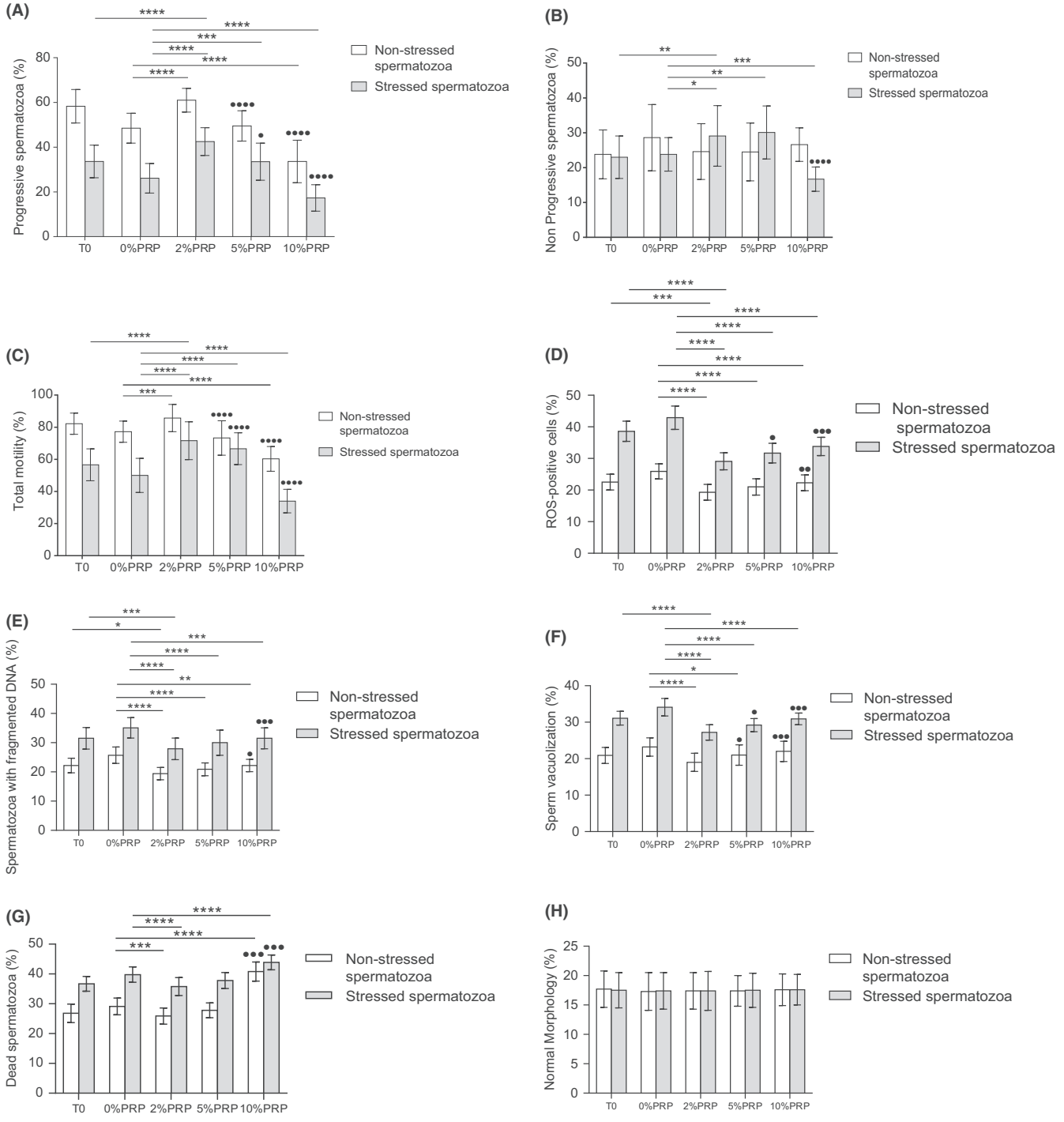


Figure 3 Effect of autologous platelet-rich plasma (PRP) on sperm motility (A-C), ROS levels (D) DNA fragmentation (E), vacuolization (F), viability (G), and morphology (H). Spermatozoa collected from 30 healthy individuals were incubated for 1 h in the presence or absence of 10 μM H_2O_2. After removal of H_2O_2, samples were washed and co-cultured with 150 μL of sperm medium in 96-well plates in the presence or absence of increasing concentrations of PRP (2, 5, and 10%). After 24-h incubation at 37 °C, sperm parameters were assessed and compared to the control group (0%PRP) and to the fresh semen (T0). Differences among groups were evaluated using the non-parametric one-way ANOVA test further tested by Tukey test for post hoc pairwise comparisons. Data are represented as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ ****$p < 0.0001$. ˙$p < 0.05$; ¨$p < 0.01$; ˙˙˙$p < 0.001$ ¨¨¨$p < 0.0001$ for differences between spermatozoa treated with 5 or 10% PRP and spermatozoa treated with 2% PRP. [Correction added on June 10, 2019, after online publication: the term spermatozoa has been corrected in Figure 3]



Finally, in both groups, sperm morphology did not change between each PRP concentration samples and controls, hence showing no effect of PRP on the appearance of the spermatozoa (Fig. 3H).

PRP-treated vs. fresh sperm

After taking into consideration all tested sperm parameters, the 2% PRP concentration was established as the optimal

condition to improve sperm quality and function. Parameters of 2% PRP-treated samples were then compared to those of fresh semen (T0).

PRP treatment had no effect on the percentage of progressive and total motility of fresh non-stressed spermatozoa. However, cells exposed to H_2O_2 and treated with 2% PRP had significantly better progressive and total motility profiles when compared to fresh stressed spermatozoa ($p < 0.0001$) (Fig. 3A,C). These results show that infertile patients with high OS levels can benefit from a 24-h PRP treatment of their collected spermatozoa, which can improve their motility and subsequently their function and quality.

Results regarding ROS levels and DNA fragmentation were comparable. Indeed, in the non-stressed group, the percentages of both studied parameters were significantly lower in 2% PRP-treated spermatozoa vs. fresh spermatozoa ($p < 0.001$ and $p < 0.05$, respectively). The same result was observed in the stressed group but with a stronger significance ($p < 0.0001$ and $p < 0.001$, respectively), hence highlighting the beneficial effect of PRP, more particularly when OS is induced (Fig. 3D,E).

As for sperm vacuolization, the percentage was similar between PRP-treated and fresh non-stressed spermatozoa. However, when H_2O_2 was exerted on sperm specimens, 2% PRP-treated samples had significantly a better vacuolization profile when compared to fresh non-treated spermatozoa ($p < 0.0001$) (Fig. 3F).

Finally, in the non-stressed and stressed groups, the percentage of dead spermatozoa did not differ between 2% PRP-treated and fresh samples, thus showing that PRP treatment prevents cellular death of spermatozoa after 24 h of incubation (Fig. 3G). Likewise, sperm morphology did not change between PRP-treated and fresh sperm, cultured in the presence and absence of H_2O_2 (Fig. 3H).

DISCUSSION

The male factor is considered a major contributory determinant to infertility. Apart from the conventional causes of sperm dysfunction, OS has been identified as an important etiology for male infertility. Among new therapies emerging in the medical field, the use of autologous PRP in human reproduction appears to be a safe treatment option with a variety of potentially beneficial effects (Lubkowska *et al.*, 2012). In this regard, the objectives of this study were first to study the involvement of OS in male infertility by evaluating the effect of H_2O_2 on various sperm function characteristics and second to explore for the first time the potential effect of PRP on human sperm parameters. Therefore, the strength of this work resides in the originality of the study design, findings, and implications in the human reproduction field. Findings from the present study showed that autologous PRP improves all sperm parameters, except cell morphology, more so in the presence of an H_2O_2-induced oxidative stress environment.

The concentrations of H_2O_2 required for the induction of OS in sperm cells are debatable. Our results are in accordance with previous reports showing that even small H_2O_2 concentrations (2.5–20 μM) can generate oxidative stress, and consequently cause remarkable alterations in sperm parameters (Ramos & Wetzels, 2001; Mahfouz *et al.*, 2008; du Plessis *et al.*, 2010). Indeed, intracellular ROS levels increased in a dose-dependent manner in spermatozoa exposed to H_2O_2. Furthermore, acute exposure of spermatozoa to an H_2O_2 concentration as low as 10 μM was enough to induce a significant decrease in sperm vitality and motility, accompanied with an increase in the percentage of vacuolization and DNA fragmentation, but without causing high death rate in cells, as shown with the 20 and 40 μM concentrations.

An excessive ROS production was shown to promote both caspase activation and apoptosis, hence resulting in cell death and reduced sperm count (Mahfouz *et al.*, 2010). Moreover, pathological high-ROS conditions have been linked to lipid peroxidation (LPO) of the sperm plasma membrane, resulting in a loss of membrane fluidity, structure, and function, and subsequently reduction of sperm motility and its ability to fuse with the oocyte (Allamaneni *et al.*, 2005; Mahfouz *et al.*, 2010). Another explanation for the cause of reduced sperm motility is that when H_2O_2 diffuses across the cell membrane, it may temporarily inactivate the glucose-6-phosphate dehydrogenase, a cytosolic enzyme that utilizes the hexose monophosphate shunt to produce the cofactor NADPH. The resulting decreased NADPH levels will limit the antioxidant-defense mechanism of spermatozoa, as this cofactor is known for its role in the reduction of glutathione and the stabilization of catalase, and consequently in the detoxification and neutralization of excessive production of ROS (Agarwal *et al.*, 2014).

On the other hand, over-production of ROS has been related to sperm DNA damage, by causing DNA strand breaks, DNA cross-links, and chromosomal rearrangements, hence compromising the paternal genomic contribution to the embryo (Agarwal *et al.*, 2017). Several authors have speculated that vacuoles could be a morphological evidence of sperm DNA fragmentation (Berkovitz *et al.*, 2006; Hazout *et al.*, 2006; Garolla *et al.*, 2008; Oliveira *et al.*, 2010; Boitrelle *et al.*, 2011; Perdrix *et al.*, 2011; Franco *et al.*, 2012; Hammoud *et al.*, 2013). Moreover, Tran *et al.* have revealed that spermatozoa with one or more vacuoles covering more than 20% of the nuclear surface had more DNA fragmentation compared to those that covered less (Tran *et al.*, 2018). In line with these data, two studies conducted by Brassesco *et al.* and Wirleitner *et al.* also showed that antioxidant treatment reduced the presence of vacuoles in the spermatozoa nucleus, most probably, because the antioxidant therapy decreases ROS production, thus supporting the relation observed in our study between sperm nuclear vacuoles and H_2O_2-induced OS (Brassesco *et al.*, 2011; Wirleitner *et al.*, 2012).

The biological role of autologous PRP in male infertility treatment was assessed by comparing parameters of the non-treated spermatozoa to those treated with 2% PRP. Interestingly, the latter group showed an improvement in vitality and motility, and a significant reduction in vacuolization, DNA fragmentation, and ROS levels. Furthermore, 2% PRP treatment enhanced sperm parameters and prevented cell death in H_2O_2-exposed spermatozoa as compared to freshly collected semen.

The beneficial effect of PRP is mainly related to its numerous bioactive components (Appel *et al.*, 2002; Laskaj *et al.*, 2009; Castillo *et al.*, 2011; Magalon *et al.*, 2014). Until present, many of these factors were studied solely on the spermatozoa and showed a significant positive effect on its quality and function.

Indeed, it has been shown that sperm incubation with IGF-1 leads to a significant decrease in DNA fragmentation (Susilowati *et al.*, 2015). The molecular mechanism of IGF-1 resides in its ability to regulate proliferation and survival of multiple cell types

through regulation of the mitochondrial cytochrome c/caspase pathway (Li *et al.*, 2003; Delafontaine *et al.*, 2004; Li & Geng, 2010). In line with its mode of action, data from a study conducted by Mathias Abiodun Emokpae *et al.* demonstrated that elevated levels of caspase 3 and cytochrome c were observed in seminal plasma from oligospermic males compared with controls, thus indicating that mitochondrial-dependent apoptotic signaling pathway may lead to oligospermia (Abiodun *et al.*, 2016).

Regarding VEGF, its presence was related to an enhancement in sperm motility, without having an effect on survival, in a concentration-dependent manner (Iyibozkurt *et al.*, 2009). Previous studies have shown that VEGF prevents oxidative damage *via* activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway *in vitro* (Tohidnezhad *et al.*, 2014). In accordance, Kensler *et al.* as well Nakamura *et al.* demonstrated that the Nrf2/antioxidant response element (ARE) signaling pathway and its regulated antioxidant enzymes play crucial roles in cellular OS defense during spermatogenesis and fertilization (Kensler *et al.*, 2007; Nakamura *et al.*, 2010).

As for FGF, Saucedo *et al.* evaluated the expression, functionality, and involvement of FGF2 in human sperm cells and have shown that, in ejaculated spermatozoa, FGFRs were localized in the acrosomal region and flagellum. The exposure of spermatozoa to FGF2 caused an increase in flagellar FGFR phosphorylation and activation of extracellular signal-regulated kinase (ERK) and protein kinase B (PKB or Akt) signaling pathways, thus leading to a significant increase in the percentage of total and progressive sperm motility. Interestingly, all responses were prevented by sperm pre-incubation with a specific inhibitor of FGFR tyrosine kinase activity (Saucedo *et al.*, 2015).

Moreover, according to the study conducted by Basciani and his colleagues, PDGF and its receptors play a leading role in testicular development and pathophysiology in humans mainly on Leydig cells affecting spermatogenesis (Basciani *et al.*, 2002). Therefore, its deficiency is highly associated with severe impairment of spermatic parameters (Gentile *et al.*, 2014).

Finally, seminal TGF-β due to its anti-inflammatory properties was found to improve sperm motility and sperm total number and to play an important role in reproductive success through interactions with the female reproductive tract after coitus (Sharkey *et al.*, 2016). On the other hand, the antioxidant Zn/Cu SOD enzyme, an important component of PRP, has been found to play a protective role in sperm motility that is closely related to sperm membrane integrity. In fact, as an important element of ROS scavenger system, its mechanism of action is based on the reduction of DNA fragmentation, resulting from H_2O_2 exposure, *via* inhibition of LPO of human spermatozoa, thus enhancing sperm motility (Perumal, 2014; Lee *et al.*, 2016; Zhao *et al.*, 2016).

All the above-mentioned factors and processes may be involved in the mechanism of PRP-dependent sperm parameter improvement. The combination of all these natural and autologous factors can reverse the negative effect of H_2O_2-induced oxidative stress and seems to have a more profound and generalized effect on sperm quality as compared to each component individually, especially that some factors such as EGF seem to have no effect on sperm parameters (Naz & Kaplan, 1993).

Limitations of this study include some constraints related to participants' recruitment and restrictions in the analysis of additional sperm parameters due to the limited number of spermatozoa in samples.

CONCLUSION

Along these lines, the new prospects on PRP may encode key factors for enhancing sperm parameters and subsequently male infertility. Referring to the sperm cell population sensitivity and the effect of H_2O_2-induced OS, the use of various compounds for supplementing the culture media next to many other antioxidants and other factors, which may be not yet addressed or known, might be of great interest. This promising strategy can be applied in assisted reproductive technologies in order to improve the outcomes of IUI and IVF and even decrease the rate of miscarriages. In this regard, further studies assessing additional parameters on a larger sample may be useful for a better understanding of the biological role of PRP in male infertility. Moreover, the investigation of the molecular mechanisms underlying PRP effect on sperm physiology would be of great interest to provide new insights for the use of this natural and safe approach in human reproduction.

CONFLICT OF INTEREST

The authors have no other conflict of interests relating to this study.

AUTHORS' CONTRIBUTIONS

RB was responsible for study design and conception, acquisition of data, analysis plan, statistical analysis, interpretation of data, drafting/revising, and final approval of article. JNI was responsible for study design and conception, data and statistical analysis, review and approval of first draft, revisions, and final version of the manuscript. MM was responsible for study design, review and approval of first draft, revisions, and final version of the manuscript. AM was responsible for study design, data analysis, review and approval of first draft, revisions, and final version of the manuscript. JA was responsible for study design and conception, review and approval of first draft, revisions, and final version of the manuscript. JA was responsible for study design, acquisition and interpretation of data, drafting, and final approval. NA was responsible for study design, acquisition and interpretation of data, drafting, and final approval.

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