Effects of Sperm Processing Techniques Involving Centrifugation on Nitric Oxide, Reactive Oxygen Species Generation and Sperm Function

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Abstract: This study was aimed at investigating the effects of sperm centrifugation on nitric oxide (NO) and reactive oxygen species (ROS) generation as well as sperm motility and viability. Human spermatozoa were centrifuged for 10 and 30 minutes (400 x g) in the presence or absence of the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME); ROS scavenger, N-(2-mercaptopropionyl)Glycine (MPG) or the combination of L-NAME + MPG. Total sperm motility was significantly decreased with 30 minutes of centrifugation whereas progressive motility and cell viability were significantly decreased with 10 and 30 minutes of centrifugation. These effects were reversed with the administration of MPG or L-NAME + MPG. Ten minutes centrifugation significantly elevated ROS and NO production ($P < 0.05$). Thirty minutes centrifugation elevated ROS generation ($P < 0.01$) whereas NO was attenuated. This study has demonstrated that 10 and 30 minutes of sperm centrifugation were detrimental to both sperm motility and viability, but generally 30 minutes centrifugation was more detrimental to sperm than 10 minutes. It has also demonstrated that 10 minutes centrifugation led to both NO and ROS elevation whereas 30 minutes centrifugation led to ROS elevation and NO attenuation. We therefore recommend that sperm separation techniques should avoid using centrifugation or prolonged centrifugation in assisted reproductive technologies.

Keywords: Motility, nitric oxide, reactive oxygen species, spermatozoa.

INTRODUCTION

The removal of sperm from the seminal fluid is a very important step in the processing of semen during assisted reproductive procedures. Most sperm processing techniques employ the use of centrifugation to separate motile sperm from non-motile or dead sperm as well as other contaminating cell debris [1]. However, sperm processing techniques that employ centrifugation have their own disadvantages. Studies have shown that sperm preparation, processing and handling lead to increased free radical generation [2]. A free radical is any compound which contains one or more unpaired electron(s) [3]. Free radicals derived from oxygen are called reactive oxygen species (ROS); examples include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\cdot}$), and hydroxyl radicals ($OH^{\cdot}$) [4-7]. Those derived from nitrogen are called reactive nitrogen species (RNS) and include nitric oxide (NO) and peroxynitrite anion ($ONOO^-$) [8, 9]. Recently, a substantial body of growing evidence suggests that excessive presence of free radicals in semen is involved in many idiopathic male factor infertility cases [10-12].

NO has become one of the most widely studied substances as of late because of its paradoxical yet important role in several biological functions. NO production is catalyzed by a family of NO synthase (NOS) enzymes [13] and has been shown to be expressed in human spermatozoa [14]. Studies have shown that exogenously applied NO had different effects on sperm function depending on the concentrations applied [15, 16]. We have previously shown that intracellular NO in human spermatozoa can be detected and measured by flow cytometry [17]. The aim of this study was to assess the effect of sperm centrifugation on NO and ROS generation as well as on sperm function (motility and viability).

MATERIALS AND METHODS

Chemicals

N^G-nitro-L-arginine methyl ester (L-NAME), N-(2-mercaptopropionyl)Glycine (MPG), propidium iodide (PI), 2,7-dichlorofluorescin diacetate (DCFH-DA) were obtained from Sigma Chemical Co., (St Louis, MO, USA); 4,5-diaminofluorescein-2/diacetate (DAF-2/DA) was from Calbiochem (San Diego, CA, USA).

Preparation of Sperm Samples

Ethical approval from our institutional review board was granted for this study. Semen was collected from normozoospermic donors according to the World Health Organization (WHO) criteria. Samples were left to liquefy for 30 minutes before processing. Fresh semen was placed in a 5ml tube and an equal amount of human tubal fluid (HTF) medium was added. The tube was centrifuged for 5 minutes at 400xg. The supernatant was discarded leaving a pellet at the bottom which was resuspended in fresh HTF medium

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and centrifuged again for 5 minutes at 400xg. The supernatant was carefully removed by aspiration without disturbing the pellet and 1.2 mL of HTF mixed with 3% bovine serum albumin (BSA) medium was layered on top of the pellet. The tube was placed on a rack inclined at 45 degrees and incubated (37˚C, 5% CO_2, 60 min). After 1 hour the media containing a homogenous motile sperm population was collected.

Centrifugation Protocol

Retrieved sperm cells were subsequently divided into aliquots of concentration $5x10^6$cells/mL. The aliquots were treated with the NOS inhibitor, L-NAME (0.7mM), the ROS scavenger, MPG (50μM) or L-NAME + MPG 30 minutes prior to centrifugation. The cells were centrifuged for either 10 or 30 minutes at 400 x g. Control means the group which was not further centrifuged after the doubled wash swim-up technique, untreated group means the group was centrifuged for either 10 or 30 minutes after the double was swim-up technique but no L-NAME and/or MPG were administered and the treated group means the group that was centrifuged for 10 or 30 minutes after the double wash swim-up method and was treated with L-NAME and/or MPG.

Motility Parameters

Motility was measured by means of computer assisted semen analysis (CASA) using Hamilton Thorne Ivos motility analyzer 120 minutes after centrifugation.

Cell Viability

Sperm cells which had received different treatments were incubated (37˚C, 5% CO_2, 120 min) and subsequently, loaded with PI (1μM, 15 min). Living cells with an intact cell membrane and active metabolism will exclude PI while cells with damaged membranes or impaired metabolism allow PI to enter the cell and stain the DNA [18]. PI fluorescence was analyzed by fluorescence-activated cell sorting (FACS).

NO and ROS Measurement

NO and ROS were measured as previously described [19]. Briefly, samples which had received different treatments were loaded with DAF-2/DA (10 μM) or DCFH-DA (5μM) and incubated (120 min, 37 ºC) in the dark. Care was taken to prevent exposure to light throughout the rest of the experimentation as the probes are light sensitive. After incubation with DAF-2/DA or DCFH-DA the cells were analysed by FACS.

Flow Cytometry

A Becton Dickinson FACSCalibur™ analyzer was used to quantify fluorescence (excitation wavelength 488 nm and emission wavelength 530 nm) at a single-cell level and data were analysed using Cellquest™ version 3.3 (Becton Dickinson, San Jose, CA, USA) software. The mean fluorescence intensity of the analysed sperm cells was determined after gating the cell population by forward and side scatter signals. In total 30 000 events were acquired. Non-sperm particles and debris were excluded by prior gating, thereby limiting undesired effects on overall fluorescence. The final gated populations usually consisted of 15 000-20 000 sperm cells.

Statistical Analysis

The results were analyzed on the GraphPad Prism™ 4 statistical program. All data are expressed as mean ± SEM. Student's *t*-test or One-way analysis of variance (ANOVA) (with Bonferroni post hoc test if $P < 0.05$) was used for statistical analysis. Fluorescence data are express as mean fluorescence (percent of control, control adjusted to 100%). Differences were regarded statistically significant if $P < 0.05$.

RESULTS

Motility Parameters

All the groups which were centrifuged for 10 minutes did not show any significant differences in total motility when compared to the control (Fig. **1**). However, 30 minutes of centrifugation significantly decreased total sperm motility in the untreated and L-NAME treated groups when compared to the control (57.67 ± 2.76%; 58.39 ± 2.56% vs. 76.17 ± 3.57%) respectively. The MPG and L-NAME + MPG treated groups differed significantly to the untreated group (67.00 ± 3.76%; 70.23 ± 4.76% vs. 57.67 ± 2.76%) which were centrifuged for 30 minutes. We also observed a statistically significant difference between the L-NAME + MPG group compared to the L-NAME only group (70.23 ± 4.76% vs. 58.39 ± 2.56%) which was centrifuged for 30 minutes.

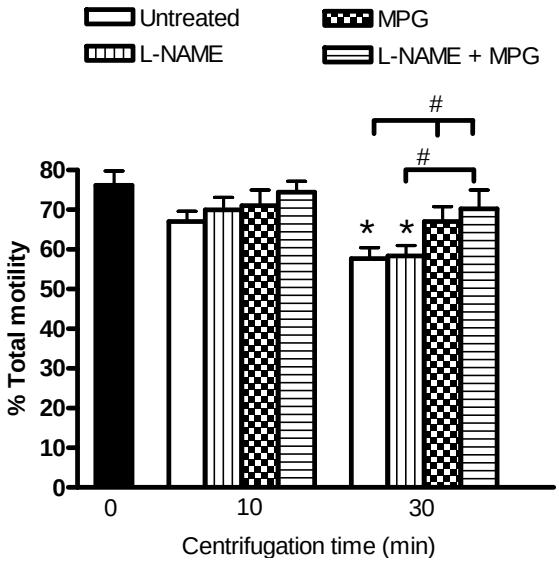

Fig. (1). Effects of 10 and 30 minutes centrifugation on total sperm motility. Spermatozoa were treated with NOS inhibitor, L-NAME; ROS scavenger, MPG; L-NAME + MPG or left untreated. *, $p < 0.05$ vs. control; #, $p < 0.05$.

Progressive motility was significantly decreased in the untreated group centrifuged for 10 minutes when compared to the control (32.83 ± 1.62% vs. 41.67 ± 1.53%). The MPG and L-NAME + MPG treated groups showed significantly higher progressive motility compared to the 10 minutes

centrifuged untreated group (40.32 + 3.23%; 41.89 + 2.34% vs. 32.83 ± 1.62%) respectively (Fig. **2**).

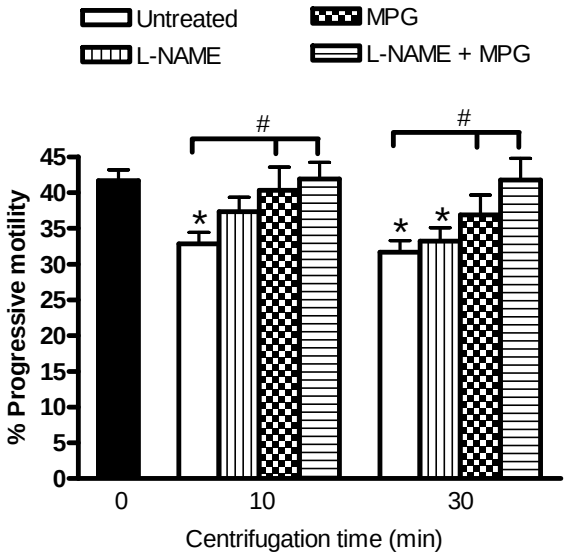

Fig. (2). Effects of 10 and 30 minutes centrifugation on progressive motility. Spermatozoa were treated with NOS inhibitor, L-NAME; ROS scavenger, MPG; L-NAME ± MPG or left untreated. *, $p < 0.05$ vs. control; #, $p < 0.05$.

For spermatozoa centrifuged for 30 minutes, we observed a significant decrease in progressive motility in the untreated and L-NAME treated groups compared to the control (31.67± 1.62%; 33.21 ± 1.87% vs. 41.67 ± 1.53%). The MPG and L-NAME + MPG treated groups' progressive motility differed significantly from that of the untreated group (36.87± 2.78%; 41.76 ± 3.05% vs. 31.67± 1.62 %;).

Cell Viability

There was a significant increase in PI fluorescence in untreated cells centrifuged for 10 minutes compared to the control (165.10±19.99 vs. control, adjusted to 100%; $P<0.05$). The 10 minutes centrifuged L-NAME treated group also showed a significant increase in PI fluorescence (157.50±12.21%) compared to the control. The 10 minutes centrifuged MPG treated group as well as L-NAME + MPG treated group showed no significant difference when compared to the control (Fig. **3**).

There was a significant increase in PI fluorescence in untreated spermatozoa centrifuged for 30 minutes compared to the control (239.40±27.78% vs. control; $P<0.001$). The 30 minutes centrifuged L-NAME treated group also showed a significant increase in PI fluorescence compared to the control (179.10±15.99% vs. control; $P<0.001$). The 30 minutes centrifuged MPG treated group as well as L-NAME + MPG treated group showed no significant difference compared to the control.

NO and ROS Production

The 10 minutes of centrifugation significantly increased DAF-2/DA fluorescence compared to the control (119.35±7.53% vs. 100%). The NOS inhibitor, L-NAME, significantly attenuated DAF-2/DA fluorescence in the 10 and 30 minutes centrifuged groups but not in the control group (Fig. **4**). On the other hand, 30 minutes centrifugation significantly reduced DAF-2/DA fluorescence compared to the control (90.68±4.5% vs. 100%).

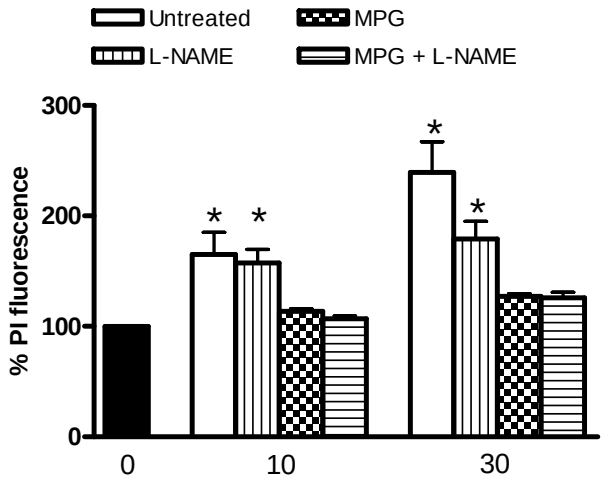

Fig. (3). Effects of 10 and 30 minutes centrifugation on PI fluorescence. Spermatozoa were treated with NOS inhibitor, L-NAME; ROS scavenger, MPG; L-NAME + MPG or left untreated. *, $p < 0.05$ vs. control.

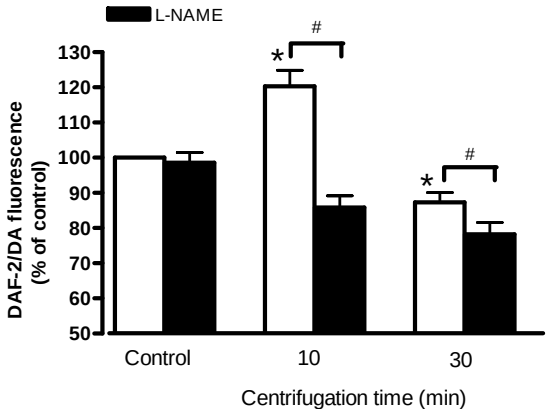

Fig. (4). Effects of 10 and 30 minutes centrifugation on DAF-2/DA fluorescence. Values are expressed as percent of control, control adjusted to 100%. *, $p < 0.05$ vs. control; #, $p < 0.05$.

Both 10 and 30 minutes centrifuged groups had significantly higher DCFH-DA fluorescence compared to the control (144.50 ± 10.53%; 153.60 ± 10.74% vs. 100%) respectively. The non-specific ROS inhibitor, MPG, significantly attenuated DCFH-DA fluorescence in the control, 10, and 30 minutes centrifuged groups (Fig. **5**). *, $p < 0.05$ vs. control; #, $p < 0.05$.

DISCUSSION

Sperm preparation for ART use is required to select and maximize the superior quality spermatozoa. The double wash swim-up technique is one of the commonly used

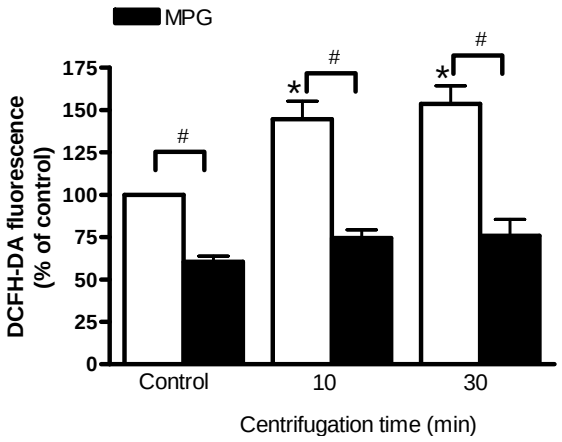

Fig. (5). Effects of 10 and 30 minutes centrifugation on DCFH-DA fluorescence. Values are expressed as percent of control, control adjusted to 100%.

processing technique to provide superior sperm quality regarding motility and normal morphology [1]. This technique employs the use of centrifugation steps. Studies have shown that sperm preparation techniques which employ centrifugation lead to ROS production [2]. This was confirmed in our study, where 10 and 30 minutes of centrifugation led to increased ROS production. ROS may play a beneficial role in normal physiological function, but once their presence is in excess they induce pathologies. They achieve this by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates and other molecules in the process causing a cascade reaction that result in cellular damage [20]. Excessive ROS lead to lipid peroxidation of the sperm plasma membrane which results in a loss of membrane fluidity which is essential for sperm motility [21]. This explains our observation of reduction in total motility, progressive motility as well as viability for centrifuged spermatozoa which were not treated with ROS scavenger.

This study also reported an increase in NO production with 10 minutes of centrifugation while 30 minutes of centrifugation attenuated its production. The decrease in NO production after 30 minutes of centrifugation might have been as a result of NOS enzyme down-regulation or loss. NO has been shown to possess antioxidant abilities by scavenging ROS [22]. An alternative explanation for its decrease after 30 minutes centrifugation could be that NO reacted with one member of the ROS family, $O_2^{-\cdot}$ to form $ONOO^-$ since we observed an increase in ROS production with 30 minutes of centrifugation.

This study has also demonstrated the importance of ROS scavengers in protecting spermatozoa from oxidative damage. The ROS Scavenger, MPG, was able to protect the sperm which was exposed to 10 and 30 minutes of centrifugation thereby restoring its motility and viability. On the other hand, lowering NO production by inhibiting NOS did not protect spermatozoa from free radicals generated due to centrifugation, thereby showing that the effect of ROS is more detrimental than that of NO generated due to centrifugation.

This study has demonstrated that 10 and 30 minutes of sperm centrifugation were detrimental to both sperm motility and viability, but generally 30 minutes centrifugation was more detrimental to sperm than 10 minutes. We therefore recommend that sperm separation techniques should avoid using centrifugation or prolonged centrifugation in assisted reproductive technologies. Future studies will indicate whether addition of ROS scavengers prior to centrifugation could improve sperm recovery in assisted reproductive technologies.

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