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APPLICATION NOTES

Effect of various commercial buffers on sperm viability and capacitation

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Abstract

A wide variety of sperm preparation protocols are currently available for assisted conception. They include density gradient separation and washing methods. Both aim at isolating and capacitating as much motile sperm as possible for subsequent oocyte fertilization. The aim of this study was to examine the effects of four commercial sperm washing buffers on sperm viability and capacitation. Semen samples from 48 healthy donors (normal values of sperm count, motility, morphology, and volume) were analyzed. After separation (density gradient 40/80%), sperm were incubated in various buffers then analysed for reactive oxygen species (ROS) production, viability, tyrosine phosphorylation (Tyr-P), cholera toxin B subunit (CTB) labeling, and the acrosome reaction (AR). The buffers affected ROS generation in various ways resulting either in rapid cell degeneration (when the amount of ROS was too high for cell survival) or the inability of the cells to maintain correct functioning (when ROS were too few). Only when the correct ROS generation curve was maintained, suitable membrane reorganization, evidenced by CTB labeling was achieved, leading to the highest percentages of both Tyr-P- and acrosome-reacted-cells. Distinguishing each particular pathological state of the sperm sample would be helpful to select the preferred buffer treatment since both ROS production and membrane reorganization can be significantly altered by commercial buffers.

Abbreviations: ROS: reactive oxygen species; Tyr-P: tyrosine phosphorylation; AR: acrosome reaction; NVC: non-viable cells; RLU: relative luminescence units; CTB: cholera toxin B subunit

Keywords

Acrosome reaction, capacitation, sperm ROS generation, Tyr-phosphorylation

History

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Introduction

Infertility affects 10–15% of couples trying to conceive [Gnoth et al. 2005] and assisted reproduction techniques (ART) such as intrauterine insemination (IUI) and *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) can increase the possibility of conception [Barratt et al. 2009]. Sperm preparation protocols currently available for assisted conception include density gradient separation and washing methods that are aimed at isolating and capacitating as much motile sperm as possible for subsequent oocyte fertilization [Henkel and Schill 2003].

Capacitation is a set of alterations leading to the acrosome reaction (AR), an exocytotic process mediated by hydrolytic enzymes (e.g. acrosin) that are released to allow sperm to fertilize the oocyte [Olds-Clarke 2003]. Several cellular changes occur at specific times and locations during sperm capacitation, including an increase in membrane fluidity due to lipid modifications [de Lamirande et al. 1997], an influx of Ca^{2+} [de Lamirande et al. 1997], generation of controlled amounts of reactive oxygen species (ROS) [de Lamirande and Gagnon 1993, 1995; de Lamirande et al. 1997; O'Flaherty et al. 2003], and phosphorylation of proteins on serine (Ser), threonine (Thr) [Liguori et al. 2005], and tyrosine (Tyr) residues [Aitken et al. 1998; de Lamirande et al. 1997; Liguori et al. 2005; O'Flaherty et al. 2005]. In the last decade growing interest has been devoted to redox-regulated events induced by ROS generation, involved in both sperm capacitation and Tyr-phosphorylation (Tyr-P) events [Aitken et al. 1998; Leclerc et al. 1997]. The factors that can lead to the fall in sperm fertilizing power is still under investigation.

Routine evaluations of an infertile couple often reveal no detectable abnormalities in sperm count, morphology, or motility, leading to a diagnosis of idiopathic or unexplained infertility. Recent studies showed that infertile patients' semen presents enhanced ROS production [Lavranos et al. 2012; Pasqualotto et al. 2008] indicating that introducing ROS evaluation as a new parameter for diagnosis of male factor infertility would be opportune [La Vignera et al. 2013].We have recently shown that endogenous human sperm ROS production is crucial for correct capacitation [Donà et al. 2011a], evaluated as the percentage of acrosome-reacted cells

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[Donà et al. 2011b]. Many efforts have been made to improve sperm quality by introducing many substances, such as vitamins, antioxidants, salts, and chemicals to commercial buffers for sperm washing/preparation. In view of the particular pervious exposure and susceptibility of these cells to oxidative stress, the aim of this study was to examine the effects of several buffers, commonly used in fertility centers, on sperm capacitation and viability.

Results

Figure 1 shows a series of sperm ROS generation curves obtained in the presence of different sperm washing buffers during a 180 min incubation. In a recent study we reported that the 0.05–0.1 (relative luminescence units) RLU value range was suitable for correct capacitation [Donà et al. 2011a]. The highest values of ROS generation were obtained with the SM-Cook or with all stored buffers (marked with * in Figure 1). All were incompatible with cell survival. Excessive ROS generation obtained in the presence of the storage buffers altered the sperm membranes and caused the loss of membrane integrity as shown by propidium iodide (PI) labeling (Figure 2 and Supplementary Figure 1, non-viable cells: NVC), thus compromising cell nuclei. Buffers marked with * differ from their non-marked counterparts as they were used after more than 3 weeks of storage at 4 °C after opening (Figure 1). This highlights the well-known problem on how buffers must be stored, since human serum albumin, glucose, lactate, etc. promote bacterial/fungal contamination, thus resulting in increased buffer ROS production.

Only PSW-Nidacon allowed cells to maintain correct ROS production for almost 3 hours of incubation. GB-Cook failed to let cells achieve the minimum throughout the incubation period, as did SPM-MediCult in the first hour, but with a net increase in the curve, finally reaching more than three times the optimal value. SM-Cook drastically stimulated ROS production to 0.5 RLU in the first 60–90 min of incubation, and similar behavior was observed with all stored buffers (marked with * Figure 1).

The Tyr-P pattern of PSW-Nidacon-incubated sperm showed the highest percentage of phosphorylated cells (almost twice the number compared with that obtained with GB-Cook and up to 6–7 times those with other buffers) (Figure 2). More importantly, PSW-Nidacon induced the highest number of cells with Tyr-P localized in the head (Tyr-P head) (twice the number of those with GB-Cook, and more than ten times those obtained with other buffers).

Interestingly, only with PSW-Nidacon did sperm achieve the highest percentage of capacitated cells as indicated by the AR value (67.2 ± 7.9%). PSW-Nidacon also protected cells from apoptosis (only 3.5 ± 1.4% of total cells were not viable) compared with the great number observed with SM-Cook and SPM-MediCult. GB-Cook, although to a far lesser extent, was also able to induce a significant level of AR (23.6 ± 3.3%) and to protect cells during incubation (NVC 15.7 ± 3.2%). When buffers were replaced by the corresponding stored counterparts (buffers *), the percentages of both Tyr-P and AR were drastically reduced with a net increase of the NVC values (Figure 2 and Supplementary Figure 1).

In order to better evaluate how these buffers altered the cells to be differentially responsive to the calcium ionophore A23187, the AR stimulus, cells were labelled with the fluorescently conjugated cholera toxin subunit B (CTB). It binds to the G_{m1} ganglioside, a key structural component of raft microdomains in somatic and sperm cells [Nixon et al. 2011] revealing their location in the membrane. Rafts are sterol-and sphingolipid-enriched domains which can act as highly dynamic membrane platforms for the selective recruitment/partitioning of membrane proteins [Nixon et al. 2011].

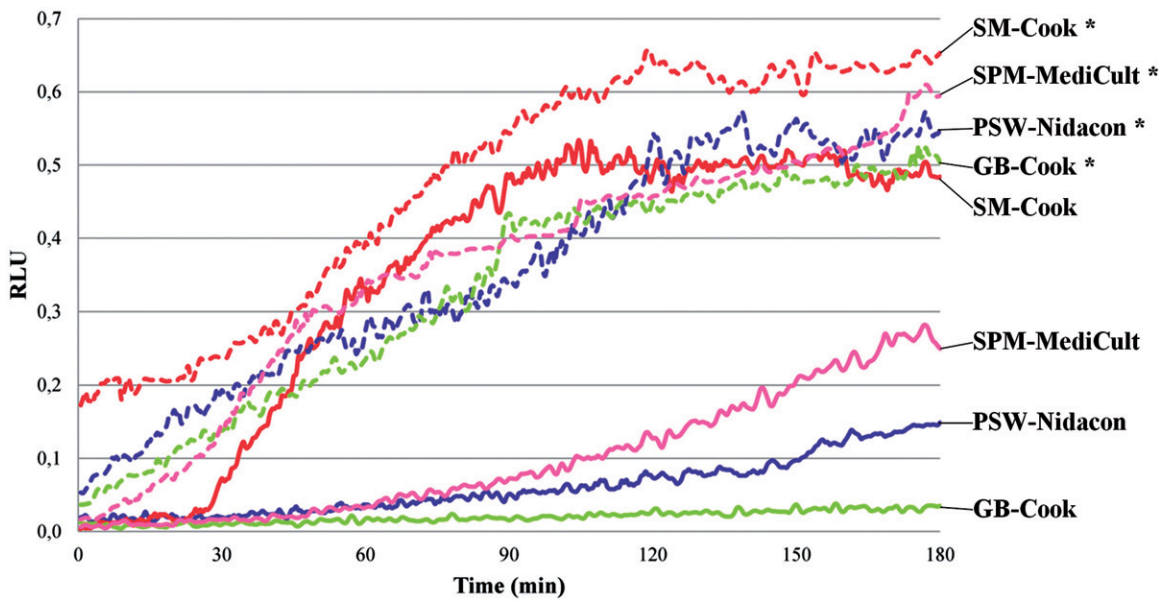

Figure 1. Time-dependent effects of different buffers' incubation on the endogenous reactive oxygen species (ROS) generation. Sperms were incubated for up to 180 min in capacitating conditions in different buffers. ROS generation was detected by monitoring luminol chemiluminescence during sperm incubation. Results are expressed as moving average of relative luminescence units (RLU)/30 seconds for 2×10^6 sperm. Detection was carried out in triplicate.

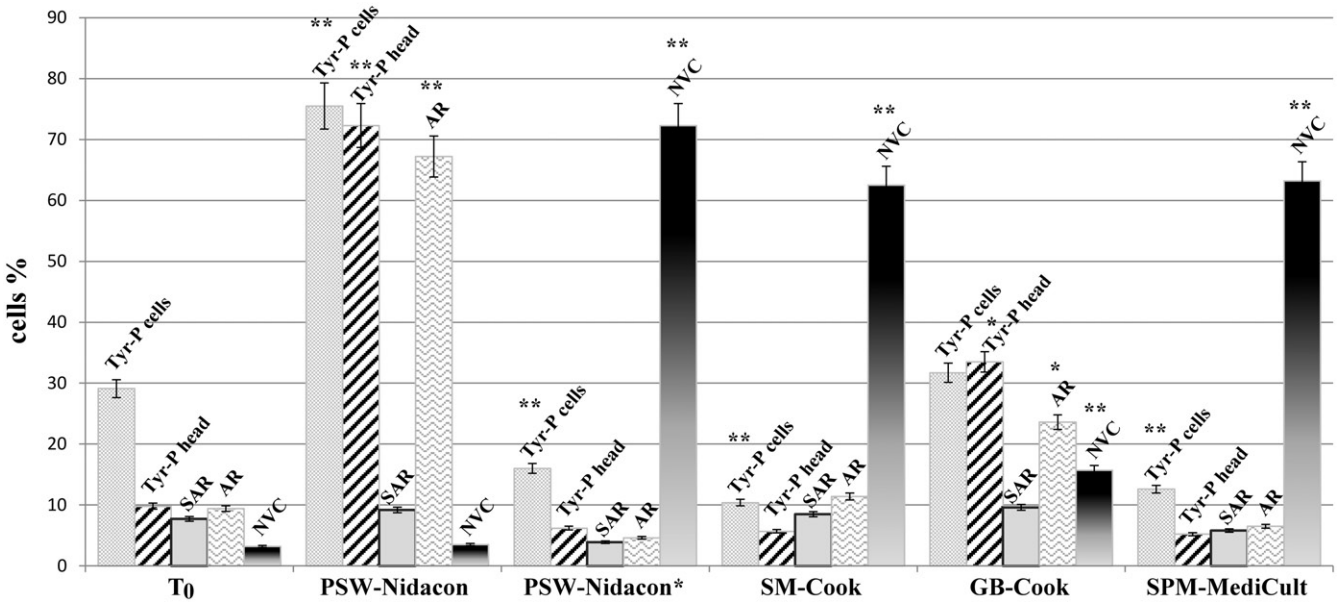

Figure 2. Sperm biochemical parameters observed with different buffers. Sperm, incubated for 180 min in capacitating conditions in different buffers, were analysed for tyrosine-phosphorylation (Tyr-P) pattern, acrosome reaction (AR), and viability (non-viable cells: NVC) by immunofluorescence cytochemistry as described in Materials and Methods. The number of cells expressed as % of the total amount of cells showing Tyr-P in any part of the cell-body, or in the head, were detected and reported as Tyr-P cells and Tyr-P head, respectively. The percentage of cells undergoing AR or NVC were also reported. Spontaneous AR (SAR) percentages were also evaluated in each sample in the absence of A23187 and values reported. $^{**}p < 0.0001$ and $^{*}p < 0.02$, comparing each sample against T_0 (ANOVA followed by Dunnett's *post hoc* test). Values are expressed as the mean ± SD.

As previously shown [Nixon et al. 2011], in T_0 (non-capacitated, control cells) CTB labeling was restricted to the flagellum (Figure 3A) in a large number of cells (77.8 ± 3.5%, panel G). Only PSW-Nidacon allowed the cell to rearrange the membrane as shown by the CTB staining of the head and peri-acrosomal regions (panel B) (54.5 ± 4.2%, $p < 0.0001$ vs. T_0, panel G), confirming that membrane rafts migrated from the flagellum to the head thus preparing sperm for the potential successive AR. PSW-Nidacon* and all the other stored buffers induced hazy CTB staining (panel C), consistent with generalized membrane denaturation and loss of integrity. SM-Cook (panel D) and SPM-Medicult (panel F), induced CTB labelling that was mainly restricted to the flagellum, in an incomplete attempt to trigger rafts translocation, which was partially achieved by GB-Cook (panel E) in 27.4 ± 2.3% of cells, as indicated by the CTB labeling of the head.

When samples were reanalyzed by CASA (Table 1) after a 180 min incubation, kinetic parameters significantly increased compared with values obtained at T_0. This confirmed that cell hyper-activation was achieved with all buffers, though a significant decrease of the percentage of motile cells was evidenced, probably due to a common gradual time-dependent slow-down [Wang et al. 1991]. It is intriguing that sperm maintained high motility even when incubated in GB-Cook and all stored buffers were not in concordance with cell viability, represented by the high percentages of NVC. This confirms that motility values are not representative of the correct status of the cells.

Discussion

Ten to fifteen percent of infertility cases are diagnosed as idiopathic, meaning that clinical and laboratory investigation of the couple were unremarkable and the cause remains to be defined. The aim of the present study was to evaluate the effects of various commercial buffers on normal sperms according to their response to AR stimulus. Sperm preparation in washing buffers is a very important step in maintaining the correct physiological activities of cells. The procedure widely adopted to assess cell viability is normally restricted to the evaluation of flagellum motility/hyperactivation, a parameter which is insufficient for sperm characterization. In this study commercial buffers were analyzed according to their capacity to induce ROS production, the Tyr-P of the head and consequently, AR. The first analysis, measurement of ROS, can rapidly classify sperm by predicting the potential percentage of cells which will be able to undergo AR. Comparing ROS values with the range expected for normal capacitation can also indicate the probable percentage of non-viable cells in the sample [Donà et al. 2011a, b].

Capacitation implies marked reorganization of membrane architecture, due to the activity of extracellular proteins which have the task of extracting cholesterol. There is growing evidence that, as in somatic cells, sperm membranes are also organized in specialized microdomains, also called rafts [Nixon et al. 2011], capable of remodeling and reorganizing themselves in the presence of extracellular proteins (in most cases, albumin), cholesterol-extracting molecules (e.g. b-methylcyclodextrin) [Choi and Toyoda 1998], bicarbonate [Botto et al. 2010], or additional compounds, such as astaxanthin, which, when inserted into the membrane bilayer, may disrupt protein–protein or protein–lipid interactions and induce membrane reorganization [Donà et al. 2013]. Once capacitation has occurred, these rafts migrate from the flagellum, where they are found extensively, to the peri-

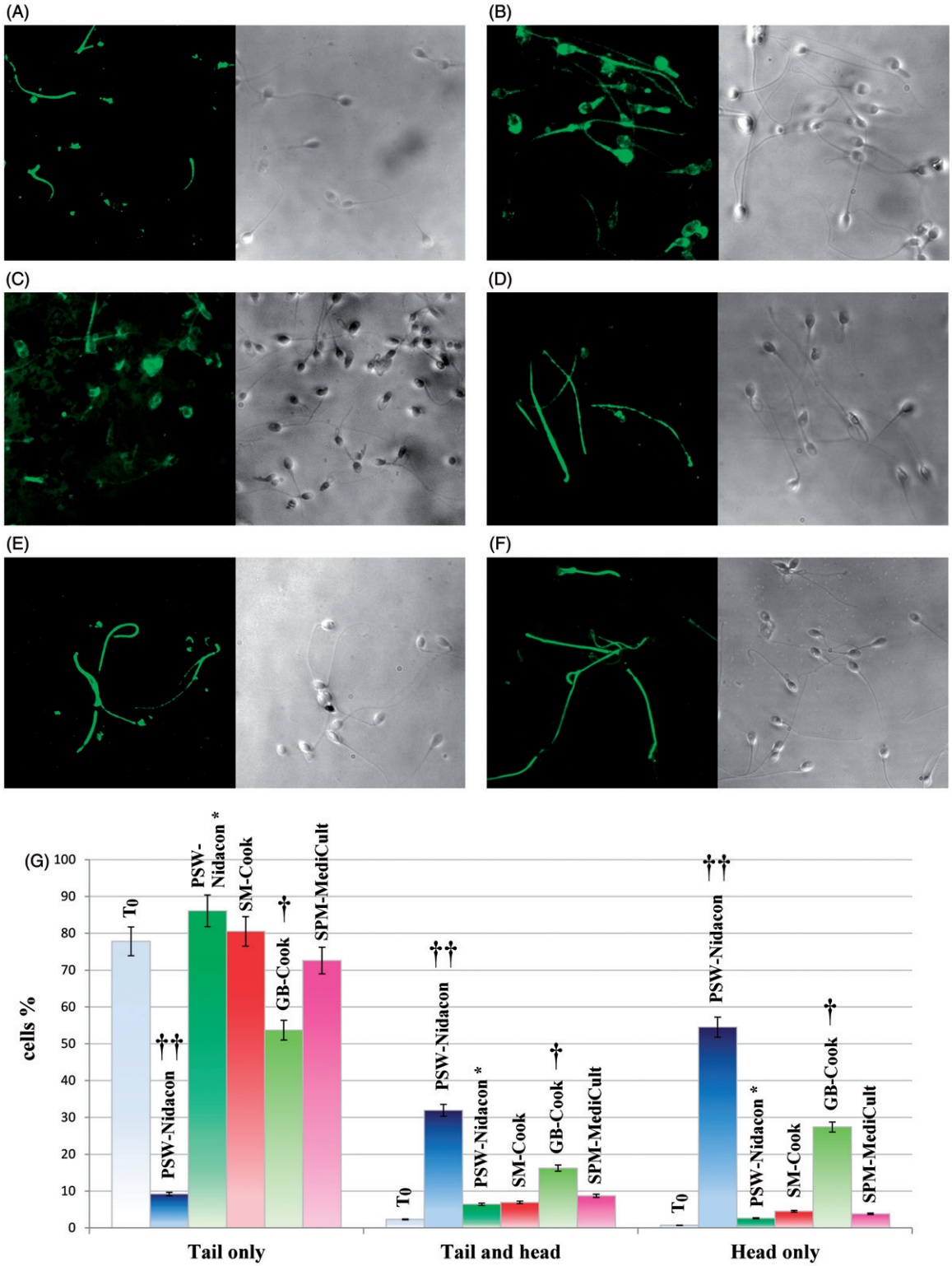

Figure 3. Localization of membrane rafts in human sperm during capacitation with various buffers. Sperms, incubated for up to 180 min in capacitating conditions in different buffers, were analyzed for CTB labeling by immunofluorescence cytochemistry as described in Materials and Methods. (A) T_0, control non-capacitated cells; sperm incubated in (B) PSW-Nidacon; (C) PSW-Nidacon* (and representative also of SM-Cook*, GB-Cook*, and GB-Cook*); (D) SM-Cook; (E) GB-Cook; (F) SPM-MediCult; (G) Number of cells expressed as % of the total amount of cells showing CTB labeling in flagellum, head, or both as transient conditions, were detected and reported. †† $p < 0.0001$ and † $p < 0.02$, comparing each sample against T_0 (ANOVA). Values are expressed as the mean ± SD.

acrosomal region [Nixon et al. 2011] where they presumably allow interaction with the oocyte. The buffers used in ART for sperm preparation must induce optimal conditions to achieve AR. Components such as, human serum albumin, glucose, lactate, etc., are important factors of capacitation but, also, the main source responsible for promoting bacterial/fungal contamination, resulting in increased buffer ROS production and the percentage of NVC. In order to avoid any complications, it

Table 1. Sperm motility and kinematic parameters observed with different buffers.

	Motility (%)	VSL (μm/s)	VAP (μm/s)	ALH (μm/s)
T_0	95 ± 2	32.4 ± 3.8	39.6 ± 3.5	1.7 ± 0.7
PSW-Nidacon	77 ± 5†	46.5 ± 3.0††	63.3 ± 6.5††	3.2 ± 0.8††
SM-Cook	64 ± 3††	42.3 ± 3.8††	61.2 ± 4.7††	2.8 ± 0.3†
GB-Cook	73 ± 4†	40.7 ± 4.1††	59.4 ± 3.6††	3.7 ± 0.4††
SPM-MediCult	75 ± 3†	38.9 ± 6.5†	56.7 ± 4.1††	3.2 ± 0.6††
PSW-Nidacon*	58 ± 6††	39.8 ± 3.5†	55.8 ± 6.2††	2.3 ± 0.6†
SM-Cook*	59 ± 7††	40.2 ± 5.3††	53.2 ± 4.8††	2.5 ± 0.5†
GB-Cook*	61 ± 6††	41.4 ± 4.3††	60.5 ± 4.7††	2.6 ± 0.4†
SPM-MediCult*	60 ± 8††	37.8 ± 4.6†	57.9 ± 6.0††	2.2 ± 0.5†

Motility and kinematic parameters of spermatozoon was evaluated with CASA at T_0 (before starting incubation) and after 180 minutes of incubation in capacitating conditions in different buffers.

Buffer* indicated stored buffer. Motility: progressive and non-progressive motility (%); VSL: straight-line velocity (μm/s); VAP: average path velocity (μm/s); ALH: amplitude of lateral head displacement(μm/s). $††p < 0.001$ and $†p < 0.05$, comparing each sample against T_0 (ANOVA). Values are expressed as the mean ± SD.

is recommendable to use fresh buffer avoiding prolonged storage. Interestingly, our results show that human serum albumin, contained in all the buffers analyzed, induces membrane rearrangement with CTB labeling of sperm heads only with PSW-Nidacon, confirming that not the single substance but the whole composition of the medium must be considered.

Collectively, on the basis of our results, membrane rearrangements are proposed to be essential for sperm to undergo AR, and the migration of rafts to the head represents the occurrence of capacitation. To achieve this goal, sperm are activated through a not yet clearly defined sequence of events, including ROS generation, which can help membrane rearrangement by directly inducing lipid peroxidation. The Tyr-P of head proteins, which may be constitutively located in sperm heads or transported by raft movements, indicates that the capacitated membrane structure has been achieved.

In this study we showed that, in normal cells, PSW-Nidacon is an optimal sperm medium to prepare sperm to undergo the potential successive AR. Concomitantly, PSW-Nidacon prevented time-dependent apoptosis (only 3.5 ± 1.4% of cells were non-viable). However, due to the specific characteristics of each buffer to allow sperm ROS generation and lipid raft relocation, using a buffer to counteract excessive/insufficient sperm ROS may still be useful in particular clinical or pathological conditions [Agarwal et al. 2012; Guerin et al. 2001]. Further studies examining the effects of each buffer in sperm clinical cases are recommended.

Materials and Methods

Semen collection and analysis

Healthy male donors ($n = 48$) (age range, 28–45 years; average age, 34.6) comprised the study population. All donors gave their written informed consent and provided detailed lifestyle histories. This study was approved by the institutional ethics committee. Semen samples were collected and analyzed as previously reported [Donà et al. 2013] and only samples with normal parameters according to the World Health Organization [WHO 2010] were processed.

Materials

Pure Sperm Wash (PSW) was obtained from Nidacon International AB (Göteborg, Sweden); Sperm Preparation Medium (SPM) from MediCult (Mollehaven, Denmark); Sperm Medium (SM), Gamete Buffer (GB), and 40/80 Gradient Kit (GK) were purchased from Cook (Queensland, Australia). When analyzed after more than 3 weeks of storage at 4 °C after opening, all buffers were marked in the text with * (PSW-Nidacon*, SPM-Medicult*, SM-Cook*, and GB-Cook*). Antibodies were from Upstate (Becton Dickinson Italia SpA., Milan, Italy) and Santa Cruz Biotechnology (Heidelberg, Germany), as described in [Donà et al. 2013], and all reagents were from Sigma-Aldrich (Milan, Italy).

Sample preparation and analysis

Sperm from the bottom pellet (80%) of a discontinuous density gradient were recovered [Donà et al. 2011b], collected in threes, and divided into four aliquots, which were separately washed with the different sperm washing buffers freshly open or after 3 weeks of storage at 4 °C (marked with *), and re-analyzed for concentration, motility, viability, and morphology; the concentration was adjusted to 80×10^6 sperm cells/ml. Aliquots, incubated in a humid chamber at 37 °C, were taken for analysis of ROS production (2×10^6 cells) and, immediately (T_0) or 180 min later, also for Tyr-P labeling (20×10^6 cells) and AR (20×10^6 cells). Luminol ROS detection, and confocal microscopy anti-P-Tyr and AR were carried out according to Donà et al. [2011b].

Evaluation of membrane raft

The membrane raft marker, GM1 ganglioside, was visualized in live human spermatozoa by staining with the cholera toxin subunit B (CTB)-FITC which displays high affinity for G_{m1} ganglioside, a key structural component of raft microdomains in somatic and sperm cells [Nixon et al. 2011]. For this purpose, suspensions of cells without incubation (T_0 as control) or variously incubated as described in sample preparation, were mixed with an equal volume of CTB (50 μg/ml) and incubated for 15 min at 37 °C. The sperm cells were then washed twice in PBS before being fixed in 4% paraformaldehyde for 30 min, mounted on poly-L-lysine coated glass microscope slides, and viewed using the confocal microscope. For each treatment, at least 200 cells were counted and categorized into three different fluorescent patterns (tail only, head and tail, or head only).

Statistical analysis

Results are expressed as means ± SD. One-way analysis of variance (ANOVA) for independent samples was applied for multiple comparisons; Dunnett's *post-hoc* test (two-tailed) was used for comparison of measured sperm parameters between T_0 and each buffer. A p value <0.05 was considered statistically significant. All statistical analyses were performed with JMP® 10 software (SAS Institute, Cary, NC, USA).

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Declaration of interest

The authors report no conflicts of interest.

Author contributions

Acquired data: AA, GD; Contributed critically for conception and design, gave important analysis and interpretation of data, drafted the manuscript and approved the final version to be published: AA, GD, GA, GB, MB, EC, GC, DA, LB.

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