Proteomics and Biomarker Identification in Improved Sperm Motility Parameters After 4 h of Ejaculatory Abstinence

Dale M. Goss, Bashir Ayad, Maré Vlok, Suzél M. Hattingh, Gerhard van der Horst, and Stefan S. du Plessis

Introduction

Increased ejaculatory abstinence (EA) is commonly used as a cost-effective mechanism of increasing the total sperm count (T.S.C.) in the semen of patients providing sperm for assisted reproductive technologies (ART) (Bahadur et al. 2016). Therefore, manipulating the duration of EA has notable effects on basic semen parameters, including sperm concentration, motility and seminal plasma constituents. These factors remain primary predictors for male infertility diagnosis, despite interindividual variations in semen characteristics. As stated by the prescribed guidelines of the World Health Organization (WHO), subjects must remain abstinent for a minimum period of 48 h, but not longer than 7 days prior to collecting a sample for a standard semen analysis (WHO 2010), yet many publications referring to these recommendations are often contradictory (Comar et al. 2017; Raziel et al. 2001; Scarselli et al. 2019). Recent studies have contributed to the inconclusive and inconsistent nature of EA by suggesting EA periods of 4 h or less, as a strategy to improve

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D. M. Goss · B. Ayad · S. M. Hattingh · G. van der Horst
Division of Medical Physiology, Stellenbosch University, Tygerberg, South Africa

M. Vlok
Department of Biochemistry and LCMS-Central Analytical Facility, Stellenbosch University, Tygerberg, South Africa

S. S. du Plessis (✉)
Division of Medical Physiology, Stellenbosch University, Tygerberg, South Africa

Department of Basic Medical Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, UAE
e-mail: Stefan.duplessis@mbru.ac.ae

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sperm quality from a functional perspective (Alipour et al. 2015; Ayad et al. 2018b; Bahadur et al. 2016; Lehavi et al. 2014). In a previous study conducted in this laboratory, we observed enhanced sperm total motility and progressive motility after only 4 h of EA compared with 4 days (Ayad et al. 2018b). Similarly, Bahadur et al. (2016) found that only 40-min EA improved sperm motility and kinematics, and Alipour et al. (2017) also observed improvements in sperm motility, velocity, progressiveness and hyperactivation after only 2 h of EA. Additionally, Mayorga-Torres et al. (2015) observed that daily successive ejaculations over a 2-week period did not negatively influence sperm concentration, motility, morphology, vitality and other functional sperm parameters. Agarwal et al. (2016) found that normospermic men providing semen samples after 1, 2, 5, 7, 9 and 11 days of EA had lower levels of sperm DNA fragmentation as EA period was reduced.

These findings also extend into clinical ART where studies have shown significantly higher pregnancy rates when using sperm from men who abstained for 1–3 h (Li et al. 2018; Shen et al. 2019), 24 h (Borges Jr et al. 2018) and 2 days or less (Marshburn et al. 2010), despite the reduced sperm concentration, T.S.C. and semen volume compared to longer EA periods. Furthermore, Scarselli et al. (2019) found little difference in sperm concentration, motility and morphology yet it was translated into producing better quality blastocysts from a genetic perspective, with significantly higher euploidy rates when using sperm from men who abstained for 1 h compared to longer periods of EA. Borges Jr. et al. (2018) attributed these observations to the detrimental effects which EA periods of greater than 4 days have on sperm DNA, and how this directly reduces the pregnancy rates from intracytoplasmic sperm injection (ICSI) cycles. Marshburn et al. (2010) however, initially did not attempt to explain the physiological reasoning behind the observed increases in pregnancy rates from male partners who underwent less than 2 days of EA. However, a subsequent study performed by the same clinic did identify higher total antioxidant capacity (TAC) after shorter periods of EA (<1 day), which could diminish oxidative damage via an alternative mechanism which does not influence sperm membrane lipid peroxidation (Marshburn et al. 2014). This sentiment was reiterated by Shen et al. (2019), in explaining increased implantation, clinical pregnancy and live birth rates using sperm from male partners having undergone 1–3 h of EA.

Ejaculated spermatozoa are terminally differentiated cells; therefore, the main functional changes they undergo are invoked by the immediate seminal plasma microenvironment and the subsequent conditions of the female reproductive system (Elzanaty et al. 2005; Said et al. 2009). This seminal plasma microenvironment is imperative as sperm are the only cells which perform their functions outside of the body (Baccetti 1984). Consequently, epididymal and accessory sex gland secretions, which constitute the seminal plasma, are of particular interest in this study. The primary function of the prostate gland is to produce and release large amounts of ionised derivatives of citric acid in the form of citrate into the prostatic fluid (Kavanagh 1994). Furthermore, the prostate releases a variety of other molecules which play important roles in fertilisation. For example, prostate-specific antigen (PSA) is a glycoprotein enzyme capable of liquefying the seminal coagulum, thereby allowing sperm to swim freely (Balk et al. 2003). Zinc released by the

prostate aids in sperm DNA stabilisation, and low levels of seminal zinc are correlated with low fertility rates (Canale et al. 1986). The seminal vesicles serve as the main producers of fructose and glucose, which are largely accepted as being the primary sources of ATP for human spermatozoa (Mann 1946; Mukai and Okuno 2019; Westhoff and Kamp 1997). This has been reiterated by showing that glycolysis in the principal piece of the flagellum is critical for normal sperm motility (Turner 2003). Seminal vesicles also release amino acids, flavins, phosphorylcholine, prostaglandins, vitamin C and a range of proteins and enzymes (Mann 1946; Schoenfeld and Numeroff 1979). These molecules released by the seminal vesicles have a variety of roles in sperm maturation and in the acquisition of fertilising potential. The epididymis is primarily responsible for the release of neutral alpha-glucosidase (NAG) (Cooper et al. 1990), as well as proteins that prevent the premature onset of capacitation during epididymal transit and storage (Fraser et al. 1990). The relationship between NAG and sperm motility remains unclear because several studies have shown positive correlations between seminal concentrations of NAG and sperm motility (Elzanaty et al. 2002; Fourie et al. 1991; Viljoen and Du Plessis 1990) and others have reported negative correlations (Guerin et al. 1990; Krause and Bohring 1999). Consequently, changes in the ratios of selected epididymal and accessory sex gland biomarkers in seminal plasma have been widely used as clinical reflections of global changes in the volume of secretions of each of these glands (WHO 2010).

Apart from the above-mentioned constituents of seminal plasma, many studies have focused on identifying key proteins linked to infertility and poor basic semen parameters, however understanding the proteins involved in less-studied conditions, such as the seminal plasma microenvironment after 4 h of EA, may be significant in understanding the mechanisms involved in the impact of EA on basic semen parameters. A vast array of extracellular proteins have been identified in seminal plasma with diverse effects on sperm function pre- and post-ejaculation, including the modulation of sperm motility (Baas et al. 1983; Bernardini et al. 2011; Graham 1994), viability (Ashworth et al. 1994), response to reactive oxygen species (ROS) (Hamada et al. 2013; Sharma et al. 2013a, b) and overall function (Caballero et al. 2012; Mann and Lutwak-Mann 1981). With current advances in genomics and transcriptomics, proteomics technology has become a powerful tool in the research of human physiology (Li et al. 2018) to identify and categorize new biomarkers for diagnosis, prognosis and treatment (Aebersold and Mann 2003; Binz et al. 2003). Furthermore, proteins identified in seminal plasma are important in the capacitation of the spermatozoa, modulation of the immune responses in the uterus, the formation of the tubal sperm reservoir (Evans and Kopf 1998; Jansen et al. 2001) and ultimately in both the sperm–zona pellucida (ZP) interaction and the sperm and oocyte fusion process (Primakoff and Myles 2002; Yi et al. 2007). Despite reflecting sperm mitochondrial activity reduction, acrosome damage and DNA fragmentation, the seminal plasma proteome directly reflects spermatogenesis and epididymal maturation status in normozoospermic men (Intasqui et al. 2016). Approximately 10% of the proteins identified in seminal plasma are of testicular or epididymal origin, while the rest originate from accessory sex glands and other parts of the body

(Batruch et al. 2011). Studies have shown that epididymal proteins play a key role in sperm quality and that proteins directly associated with testicular function may be found in seminal plasma (Intasqui et al. 2013; Milardi et al. 2012; Pilch and Mann 2006; Wang et al. 2009). Due to the wide variation of seminal plasma protein origin and functions, seminal proteins could potentially be used for diagnosis and monitoring of pathways and interactions affecting male fertility, such as non-obstructive azoospermia, obstructive azoospermia, asthenozoospermia, varicocele and vasectomy (Batruch et al. 2011; Drabovich et al. 2011; Heshmat et al. 2008; Intasqui et al. 2013; Wang et al. 2009; Zylbersztejn and Fraietta 2013). Within the rapidly evolving field of proteomics, the establishment of extensive databases of proteins specific to spermatozoa and seminal plasma allows for advanced research into male infertility, extending to scenarios such as diagnostics and subsequent treatment approaches in idiopathic cases (Oliva et al. 2009).

For the purpose of the present study, basic semen parameters including sperm kinematics were selected as adequate measures of semen and sperm quality. Furthermore, citric acid, NAG and fructose were selected as key biomarkers of prostate, epididymal and seminal vesicular secretion and function, respectively. Additionally, possible direct effects of variations in the concentrations of these biomarkers on sperm functional improvements were explored along with the proteomic profile of the seminal plasma microenvironment and how these proteins may interact with the sperm in pre- or post-ejaculation when comparing samples after 4 h and 4 days of EA.

Materials and Methods

Prior to the beginning of the study, ethics approval was obtained from the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences at Stellenbosch University. Informed written consent was provided by all subjects, and the study was performed in accordance with the Declaration of Helsinki (World Medical Association 2014). Freshly ejaculated semen samples were collected from 16 consecutive healthy donors (aged 19–25 years) at the laboratories of the Stellenbosch University Reproductive Research Group (SURRG). All samples were collected according to WHO (2010) guidelines in a location close to the laboratory and received within 10 min of collection. The first sample from each donor was collected after an EA period of 4 days; the second sample was collected from the same donor 4 h subsequent to the first collection. Samples were immediately placed in an incubator (37 °C, 5% CO_2, 30 min) and allowed to fully liquefy before further processing. Donor information including identification, age, date of semen collection, time of semen collection, abstinence duration, sample volume, pH, appearance, colour, odour, liquefaction, viscosity and agglutination was recorded. Sperm were isolated from the semen using double-wash method and seminal plasma was frozen and stored in liquid nitrogen for biochemical analyses. Sperm concentration, motility, kinematic and velocity parameters were determined using computer-aided

sperm analysis (CASA) with a Sperm Class Analyser version 5.4 (SCA 5.4, Microptic SL, Barcelona, Spain). The kinematic parameters (see Supplementary Table 1) measured were as follows: curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR) and Wobble (WOB).

Seminal plasma citric acid, fructose and NAG concentrations were measured using commercially available assay kits (Citric Acid, Fructose and Episcreen plus assay kits respectively; FertiPro) according to the manufacturer's instructions. Absorbance values were measured spectrophotometrically for citric acid and NAG at 405 nm and fructose at 470 nm using a FLUOstar Omega microplate reader (BMG Labtech). Citric acid and fructose concentrations are expressed in milligrams per millilitre, whereas NAG activity is expressed as milli-international units per millilitre.

A detailed description of the proteomic analyses is available as Supplementary Methods. The proteomic analyses of seminal plasma and the protein concentration of each of the 16 samples were measured using a Direct Detect® Infrared Spectrometer. Samples were then pooled in groups of four where each sample contributed an equal amount of protein (240 µg). SDS-PAGE was then performed on each group and gels were stained with "Coomassie brilliant blue" and subsequently washed with 25% methanol. Gels were sliced and de-stained with 200 mM NH_4HCO_3:acetonitrile 50:50 (Sigma-Aldrich, St. Louis, USA). In-gel digestion was performed as described in Supplementary Methods. LC-MS/MS was performed on a Thermo Scientific™ Ultimate™ 3000 RSLC nano and a Thermo Scientific Fusion Tribrid mass spectrometer equipped with a Nanospray Flex ionization source (Thermo Fisher Scientific, Waltham, USA).

Statistical Analyses

Comparisons between parameters measured after 4 days and 4 h of EA were performed using both Students paired *t*-test for normally distributed data sets and Mann-Whitney U-tests for nonparametric data sets, on GraphPad Prism© version 7.00 for Windows (GraphPad Software, La Jolla California, USA) and StatAdvisor™ (Statpoint Technologies, Inc., Warrenton Virginia, USA) for two sample paired comprehensive tests (*t*-test, signed-rank test, chi-square and Mann–Whitney (Wilcoxon) W-tests). All values are presented as mean ± SE. Statistical significance was set at $p < 0.05$. The raw files generated by the mass spectrometer were imported into Proteome Discoverer v1.4 (Thermo Fisher Scientific, Waltham, USA) and processed using the Sequest algorithm. The results files were imported into Scaffold 1.4.4 and identified peptides validated using the X!Tandem search algorithm included in Scaffold. Peptide and protein validation were done using the Peptide and Protein Prophet algorithms. Protein quantitation was performed performing a *t*-test on the paired data with the Hochberg–Benjamini correction applied. Protein quantitation was performed performing a *t*-test on the paired data with the

Hochberg–Benjamini correction applied. STRING.org interaction software database was utilized for pathway analysis and protein interaction of DEPs.

Results

As indicated in Fig. 1a–c, semen volume (P = 0.025), sperm concentration (P = 0.018) and subsequent T.S.C. (P < 0.001) were significantly decreased after 4 h compared with 4 days EA. A significant increase in the percentage of sperm total motility (71.8 ± 3.5 vs. 64.7 ± 3.5%, respectively; P = 0.03) and progressive motility (58.5 ± 3.4 vs. 47.7 ± 3.6%, respectively; P = 0.001) was observed after 4 h of EA, whereas no significant difference in the percentage of static (28.3 ± 3.5 vs. 35.5 ± 3.5% after 4 h and 4 days EA, respectively) or non-progressive (13.3 ± 0.9 vs. 16.2 ± 1.0% after 4 h and 4 days EA, respectively) spermatozoa was observed (see Fig. 1d). Kinematics assessment showed that VCL (91.8 ± 6.2 vs. 78.6 ± 4.0 μm s^{-1}, respectively; P = 0.035) and VAP (58.1 ± 2.897 vs. 49.6 ± 2.2%, respectively; P = 0.006) were also both significantly higher after 4 h versus 4 days of EA (see Fig. 1e, f). Furthermore, Table 1 shows that, after 4 h versus 4 days EA, there were significantly lower concentrations of citric acid (P = 0.037), NAG (P = 0.005) and fructose (P = 0.008). Additionally, when considering T.S.C, the absolute amount of fructose per spermatozoon was significantly lower after 4 h of EA (P = 0.037), whereas there was no significant effect of duration of EA on NAG or citric acid.

A total of 2889 proteins were identified in the seminal plasma of the entire study population, of which 22 extracellular proteins being significantly upregulated after 4 h of EA, and 2 extracellular proteins upregulated after 4 days of EA (see Table 2). The GO annotations revealed their involvement in carbohydrate binding, enzyme activator activity, protein binding, GTPase and ATPase activity. The variety of biological processes and cellular locations are presented as supplementary figures (Supplementary Fig. 1a, b).

Discussion

Figure 1a–c clearly shows that semen volume, sperm concentration and T.S.C. were reduced after 4 h of EA as expected. However, interestingly, total motility, progressive motility and several sperm kinematic parameters, including VAP, VCL and the proportion of rapid sperm were significantly higher after 4 h when compared to 4 days of EA (see Fig. 1d–f). These results are in line with two large studies performed by Comar et al. (2017) and Levitas et al. (2005), who found that the duration of EA had a significant direct relationship with sperm concentration and semen volume, yet a significant indirect relationship on sperm motility. The increase in sperm concentration and semen volume was attributed to the accumulation of sperm

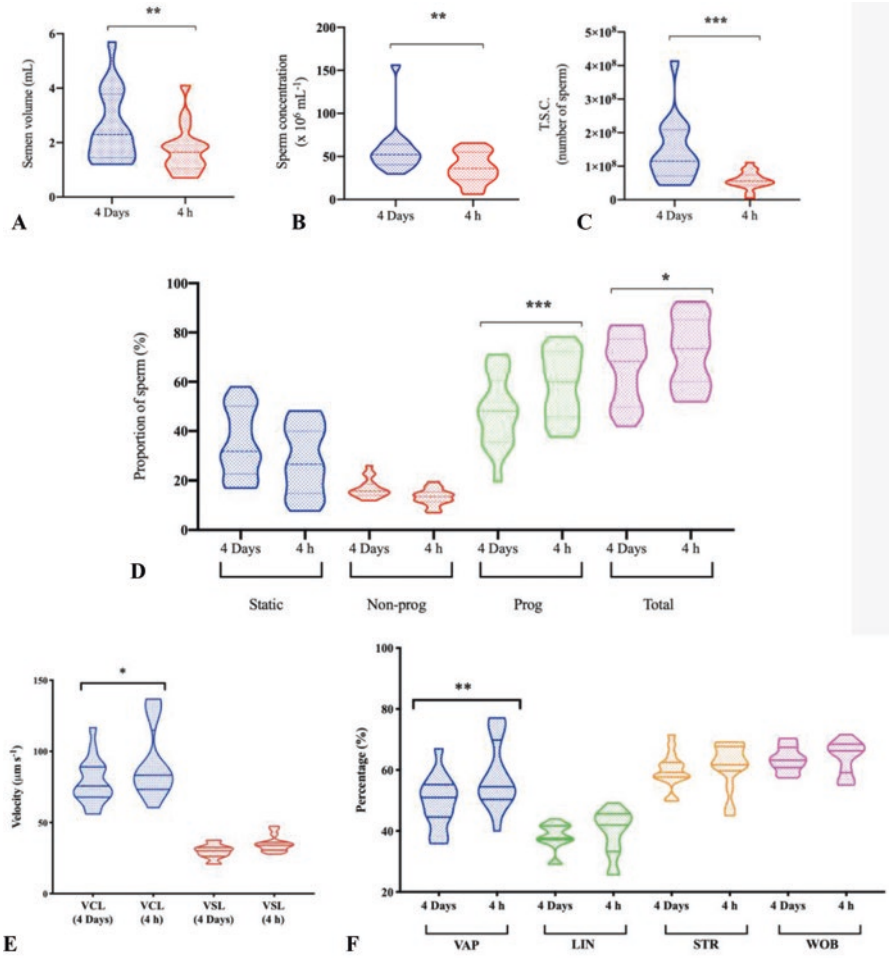

Fig. 1 Summary of basic semen parameters as measured with the Sperm Class Analyser (V5.4, Microptic) (**a**) semen volume, (**b**) sperm concentration, (**c**) T.S.C, (**d**) sperm motility parameters and (**e**, **f**) kinematics, after 4 days and 4 h of ejaculatory abstinence (EA). Data are the mean ± S.E.M. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; *T.S.C.* total sperm count. *Non-prog* non-progressive motility, *Prog* progressive motility, *total* total motility, *LIN* linearity, *STR* straightness, *VAP* average path velocity, *VCL* curvilinear velocity, *VSL* straight-line velocity, *WOB* wobble

reserves and the subsequent storage of these reserves in the epididymis, whereas the motility decreases were attributed to elevated levels of reactive oxygen species (ROS) and the ensuing DNA damage experienced by stored spermatozoa over longer periods of EA. Damaged spermatozoa (Iwasaki and Gagnon 1992) or infiltrating leukocytes (Kessopoulou et al. 1992) are the sources of ROS, which are directly correlated with reducing sperm motility parameters

Table 1 Summary of specific epididymal and accessory sex gland secretions per millilitre of seminal plasma and per spermatozoon after 4 days and 4 h of EA (Mean ± S.E.M)

Parameter	4 Days EA	4 h EA	Paired *t*-test (*P*-value)	Mann-Whitney *U*-test (*P*-value)
Concentration (mL^{-1})				
Citric Acid (mg mL^{-1})	8.01 ± 1.35	5.96 ± 1.23	–	0.037
NAG (IU mL^{-1})	29.32 ± 3.53	19.92 ± 3.61	0.005	–
Fructose (mg mL^{-1})	4.07 ± 0.52	1.66 ± 0.52	0.008	–
Absolute amount (spermatozoon^{-1})				
Citric Acid (ng $sperm^{-1}$)	0.165 ± 0.033	0.254 ± 0.059	–	ns
NAG (nIU $sperm^{-1}$)	0.056 ± 0.080	0.570 ± 0.130	ns	–
Fructose (ng $sperm^{-1}$)	0.045 ± 0.016	0.036 ± 0.011	–	0.037

EA ejaculatory abstinence, *NAG* neutral alpha-glucosidase

From the data shown in Table 1, it is clear that all the measured accessory sex gland secretions decreased after 4 h of EA, apart from citric acid concentration per millilitre and NAG per spermatozoon. These significant reductions in citric acid, NAG and fructose concentrations after 4 h of EA may be due to the insufficient time for the epididymis and accessory sex glands to produce their respective secretions, resulting in a decrease in semen volume associated with short abstinence (Goss et al. 2019; Levitas et al. 2005). When considering the quantity of each accessory sex gland secretion per spermatozoon, a significant reduction in fructose was observed. This observation contradicted the hypothesis that an increase in sperm motility should relate to an increased availability of glycolysable substrates. With variations in sperm concentration, available energy for sperm function may vary too. The primary energy source for human sperm appears to be glucose, metabolized and utilized via the glycolysis pathway, as well as pyruvate produced by this process, rather than oxidative phosphorylation. Visconti (2012) stipulated that ATP production in the individual sperm compartments is vital for various roles, such as hyperactivation and acrosome reaction. Seminal fructose and glucose are considered to be the main sources of energy by glycolytic breakdown in the principal piece of human spermatozoa, as well as being critical for normal sperm motility (Goodson et al. 2012; Mann 1946; Patel et al. 1988; Turner 2003). These glycolysable substrates are able to maintain high proportions of motile spermatozoa and increases in tyrosine phosphorylation, whereby midpiece mitochondria are supplied with pyruvate from the glycolytic breakdown of fructose and glucose (Goodson et al. 2012). It has been stated that human sperm motility relies largely, if not solely, on the glycolytic breakdown of sugars, yet the mere presence of mitochondrial machinery cannot be ignored (Rees et al. 1990; Williams and Ford 2001). Travis and Moss (1998) observed that spermatozoa maintain motility in the presence of oxidative phosphorylation un-couplers, indicating that mitochondrial respiration is essential to other processes and not primarily sperm flagellar movement. This observation was further reiterated by Miki et al. (2004). The balance between glycolytic energy production and mitochondrial oxidative phosphorylation is species-specific and

alternate between these two processes or function synergistically depending on substrate availability (Storey 2004).

As previously mentioned, these metabolic reactions are relegated to the principal piece of the spermatozoon, as the enzymes responsible for glycolysis and gluconeogenesis are concentrated in this portion of the flagellum (Eddy et al. 2003; Martínez-Heredia and Oliva 2006; Storey and Kayne 1975; Travis and Moss 1998). Oxidative phosphorylation in the midpiece also provides ATP with the ability to maintain low levels of tyrosine phosphorylation and hyperactivation in the presence of non-glycolysable substrates such as pyruvate, lactate and hydroxybutyrate and the absence of glycolysable substrates (Goodson et al. 2012).

Further analysis of seminal plasma using LC-MS/MS identified a total of 2889 unique proteins. Of these 22 extracellular proteins were upregulated after 4 h of EA, and 2 proteins upregulated after 4 days of EA (see Table 2). The large amount of

Table 2 Differentially expressed proteins from the comparison of samples from donors after 4 h and 4 days of EA (*Da* Dalton)

Accession number	Gene	Protein name	Molecular weight (Da)	*P*-value	Expression
Extracellular proteins					
Q6UW15	REG3G	Regenerating islet-derived protein 3-gamma	19,330	<0.0001	4 Days high, 4 h low
O00469	PLOD2	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	84,686	<0.0001	4 Days high, 4 h low
Q96DA0	ZG16B	Zymogen granule protein 16 homolog B	22,739	<0.0001	4 Days low, 4 h high
P62258	1433E	14-3-3 protein epsilon	29,174	<0.0001	4 Days low, 4 h high
P16035	TIMP2	Metalloproteinase inhibitor 2	24,399	0.00014	4 Days low, 4 h high
P09960	LKHA4	Leukotriene A-4 hydrolase	59,733	<0.0001	4 Days low, 4 h high
O14910	LIN7A	Protein lin-7 homolog A	25,997	<0.0001	4 Days low, 4 h high
Q99988	GDF15	Growth/differentiation factor 15	34,140	0.00028	4 Days low, 4 h high
Q8NFZ8	CADM4	Cell adhesion molecule 4	42,785	0.00042	4 Days low, 4 h high
Q8N5I2	ARRD1	Arrestin domain-containing protein 1	45,981	0.00025	4 Days low, 4 h high
Q8IYS1	P20D2	Peptidase M20 domain-containing protein 2	47,776	0.00031	4 Days low, 4 h high
Q9HBA9	FOLH1B	Putative N-acetylated-alpha-linked acidic dipeptidase	50,045	<0.0001	4 Days low, 4 h high
P11908	PRPS2	Ribose-phosphate pyrophosphokinase 2	34,769	<0.0001	4 Days low, 4 h high
P21291	CSRP1	Cysteine and glycine-rich protein 1	20,567	<0.0001	4 Days low, 4 h high

(continued)

Table 2 (continued)

Accession number	Gene	Protein name	Molecular weight (Da)	*P*-value	Expression
P19652	A1AG2/ ORM2	Alpha-1-acid glycoprotein 2	23,603	0.00013	4 Days low, 4 h high
P04433	IGKV3-11	Immunoglobulin kappa variable 3-11	12,575	<0.0001	4 Days low, 4 h high
P02511	CRYAB	Alpha-crystallin B chain	20,159	0.00013	4 Days low, 4 h high
P62979	RPS27A	Ubiquitin-40S ribosomal protein S27a	17,965	<0.0001	4 Days low, 4 h high
Q9H0R4	HDHD2	Haloacid dehalogenase-like hydrolase domain-containing protein 2	18,533	0.00029	4 Days low, 4 h high
Q8WUD1	RAB2B	Ras-related protein Rab-2B	24,214	<0.0001	4 Days low, 4 h high
O00754	MAN2B1	Lysosomal alpha-mannosidase	113,744	0.00038	4 Days low, 4 h high
P57735	RAB25	Ras-related protein Rab-25	23,496	<0.0001	4 Days low, 4 h high
P17066	HSPA6	Heat shock 70 kDa protein 6	71,028	0.00025	4 Days low, 4 h high
P20340	RAB6A	Ras-related protein Rab-6A	23,593	<0.0001	4 Days low, 4 h high
Intracellular proteins					
Q9H853	TUBA4B	Putative tubulin-like protein alpha-4B	27,551	<0.0001	4 Days low, 4 h high
P60891	PRPS1	Ribose-phosphate pyrophosphokinase 1	27,526	0.00023	4 Days low, 4 h high

proteins identified in seminal plasma highlights the wealth of information available in the constituents of the microenvironment to which post-ejaculatory sperm are exposed. Although this is essentially a snapshot into the physiological products and signals occurring in and around ejaculated spermatozoa, it may elucidate extrinsic influences and mechanisms by which sperm gain favourable traits such as improved motility and kinematics after successive ejaculations. Despite the direct availability of glycolysable substrates for sperm motility, sperm acquire motility and fertilizing capacity by interacting with a multitude of biologically active molecules throughout transit and storage in the male reproductive system. Supplementary Fig. 1a shows the biological processes of all the proteins identified by LC-MS/MS, whereby 145 and 142 proteins were involved in reproduction and reproductive processes respectively. Furthermore, a total of 815 proteins are involved in metabolic processes, whereas a total of 475 proteins are linked to developmental processes. Table 2 divides the DEPs into two distinct groups; those proteins primarily located intracellularly, which have limited scope in our analyses as they are most likely identified due to sperm and residual-cell lysis during the process of cellular preparation for analysis, and extracellular proteins such as those found in exosomes and those

released as paracrine signals in the male reproductive system, which are more important when considering the interaction between epithelial cells in the epididymis and the propagating sperm. These exosome-derived biomarkers are considered "treasure chests" filled with diagnostic molecules reflecting the status of the male reproductive system (Duijvesz et al. 2011; Poliakov et al. 2009; Simpson et al. 2009). Consequently, these secreted epididymal proteins have been extensively studied in order to improve outcomes of male infertility cases and conversely, an approach to novel modes of male contraception.

In order to explain the observed influence which EA has on basic semen parameters, we postulate four mechanisms functioning either independently or in unison, to improve sperm motility after 4 h of EA. First, the increase in sperm motility after 4 h of EA may centre on the preferable seminal plasma microenvironment created, whereby there is a reduced sperm concentration relative to the abundance of either glycolysable or non-glycolysable substrates for glycolysis and/or mitochondrial oxidative phosphorylation respectively. With fructose being significantly reduced, explaining this phenomenon from a metabolic standpoint must indicate either an abundance of another glycolysable substrate not measured (i.e. glucose) or an increase in oxidative phosphorylation of non-glycolysable substrates such as pyruvate or lactate. The ability of sperm physiology to select the preferred ATP source for maintenance of motility is not a foreign concept (Visconti 2012). Thus, with a decrease in glycolysable substrate availability in the seminal plasma, non-glycolysable substrates can still be converted to ATP by the mitochondria, thus fuelling the observed motility parameters (Bone et al. 2001; Goodson et al. 2012). Furthermore, the observed increases in sperm motility may not be due primarily to seminal plasma energy substrates, but rather the quality of the spermatozoon itself. Ayad et al. (2018a) suggested that the observed sperm functional improvements after 4 h compared with 4 days EA is due to the expulsion of degenerating and poor quality spermatozoa in the initial ejaculate when considering two successive ejaculates. In addition, as shown in Fig. 2a, it is clear that 10 of the 16 individual samples contributed more citric acid per spermatozoon after 4 h of EA, whereas Fig. 2b, c depicts a clearly lower proportional amount of fructose and NAG per spermatozoon: the contribution of 12 of 16 and 8 of 16 individual samples was proportionally less after 4 h of EA, respectively. The increases in citric acid per spermatozoon, although not statistically significant, cannot be ignored. The amount of citric acid per spermatozoon increased by a mean of 80%, indicating a possible explanation to the observed increases in sperm motility. In contrast, fructose decreased significantly by 20% after 4 h compared with 4 days EA**.** Elzanaty et al. (2005) found that shortened EA (2 days) is associated with decreased prostatic secretions and increased epididymal secretions**.** A previous study performed by Medrano et al. (2006) observed citric acid acting as a prominent substrate for energy metabolism in spermatozoa in addition to causing slight variations in seminal plasma pH; however, its relationship with and role in EA has not been adequately elucidated. An increase in the amount of available citrates for citric acid cycle utilisation in sperm metabolism may increase the energy available for sperm motility. As mentioned previously, spermatozoa use fructose originating from the seminal vesicles as carbohydrate

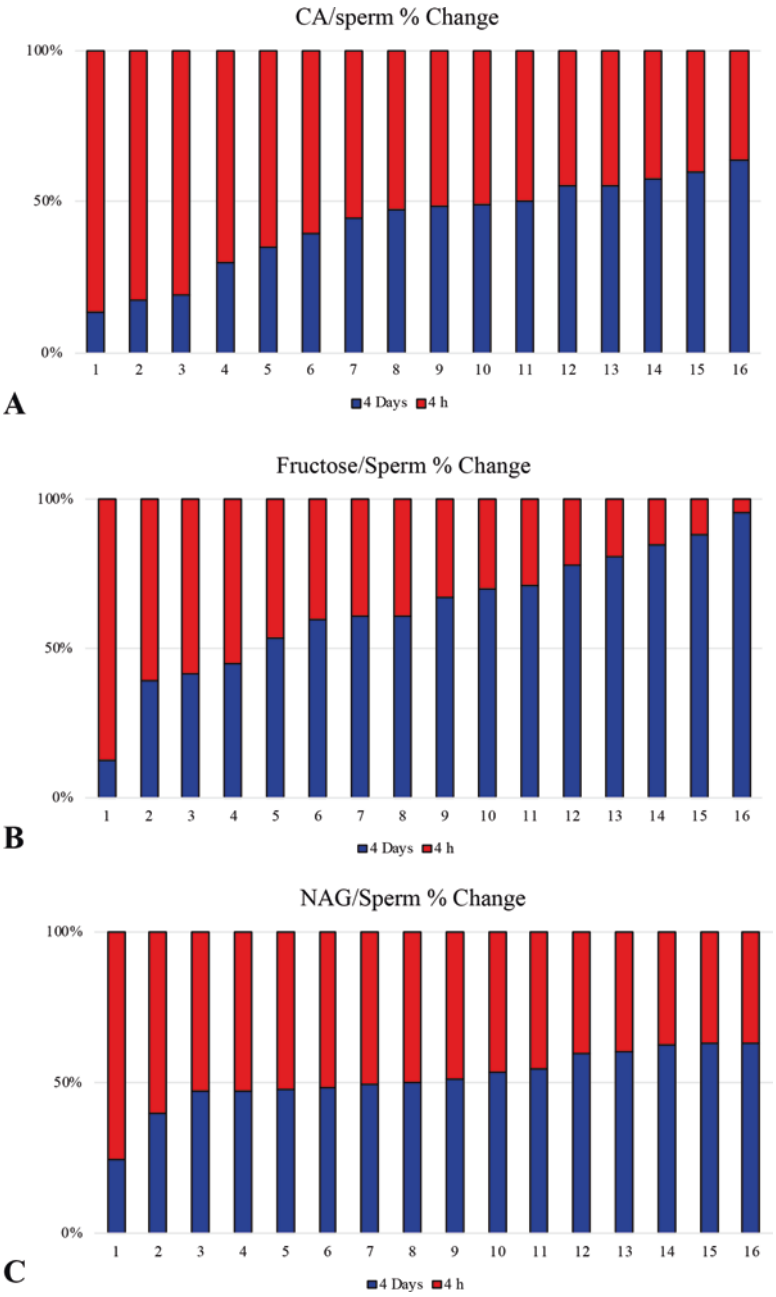

Fig. 2 Stacked columns showing percentage changes for each individual donor ($n = 16$) per spermatozoon in citric acid (CA; **a**), fructose (**b**) and neutral alpha-glucosidase (NAG, **c**). (Adapted from Goss et al. 2019)

source. Once absorbed by spermatozoa, the fructose is then metabolized into glucose and eventually converted to acetyl-coenzyme A (CoA), which drives the citric acid cycle, ultimately leading to the production of utilisable energy in the form of ATP, produced by the electron transport chain (Fig. 3). Furthermore, it has been shown that ATP production in spermatozoa preincubated in citrate increases without the addition of fructose (Medrano et al. 2006). It is believed that this occurs when citrates are converted to malate, an intermediary, that is subsequently used in the citric acid cycle in sperm mitochondria (Visconti 2012).

Finally, protein biomarkers may play an important, yet ancillary role in the observed improvements in sperm motility and kinematics after 4 h of EA when compared to 4 days. The protein interaction database analysis of DEP's in the seminal plasma from subjects after 4 h and 4 days of EA, was performed using STRING.org (see Fig. 4). Several clusters of protein interactions were identified and interestingly, of the four proteins involved in carbohydrate binding, two proteins being Zymogen granule protein 16 homolog B (ZG16B) and lysosomal alpha-mannosidase (MAN2B1) were upregulated after 4 h of EA. The other two proteins, regenerating islet-derived protein 3-gamma (REG3G) and procollagen-lysine,2-oxogluterate 5-dioxygenase 2 (PLOD2), were upregulated after 4 days of EA.

REG3G has been previously linked to acute immune response against gram-positive bacteria and not with any metabolic function in sperm (Mukherjee and Hooper 2009), and PLOD2 forms hydroxylysine residues in collagens, serving as sites for carbohydrate attachment important for the stability of collagen cross-links (Turpeenniemi-Hujanen and Kivirikko 1980). Interestingly, although ZG16B has only been linked to carbohydrate binding in the retina, MAN2B1 is pivotal in the catabolism of N-linked carbohydrates during glycoprotein turnover by cleaving all known alpha-mannosidic linkages (Gaudet et al. 2011). This may have implications in the fluidic mechanisms of carbohydrate utilization by sperm in the seminal plasma microenvironment and may highlight the ability of sperm to metabolize other monosaccharides as sources of ATP, including mannose (Mann 1967; Rodriguez-Gil 2006). Furthermore, Ras-related protein Rab-2B (RAB2B), Rab-25 (RAB25) and Rab-6A (RAB6A) are directly linked proteins which were upregulated after 4 h, these proteins are involved in GTP binding and facilitating protein transport in various cell types. These small GTP-binding proteins have been identified on human sperm acrosomal caps (Naz et al. 1992) and hamster sperm flagellum (NagDas et al. 2002). These proteins could be involved in flagellar motility through interaction with some downstream effectors, the extracellular signal-regulated kinase (ERK) pathway, protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) (NagDas et al. 2002; Naz et al. 1992). These phosphorylation events, involving PKC (Rotem et al. 1990) and ERK1/2 (Lu et al. 1999), have been positively related to the acquisition and maintenance of sperm motility. Another protein upregulated after 4 h of EA is heat-shock 7 kDa protein A6 (HSPA6), which is a heat shock protein also linked to mitogen-activated protein kinase (MAPK) cascades including ERK1/2 (Shacoski 2012). Heat shock proteins are produced in response to cellular exposure to stressful conditions, and their expression has also been directly related to sperm motility in both porcine (Huang et al. 1999, 2000) and

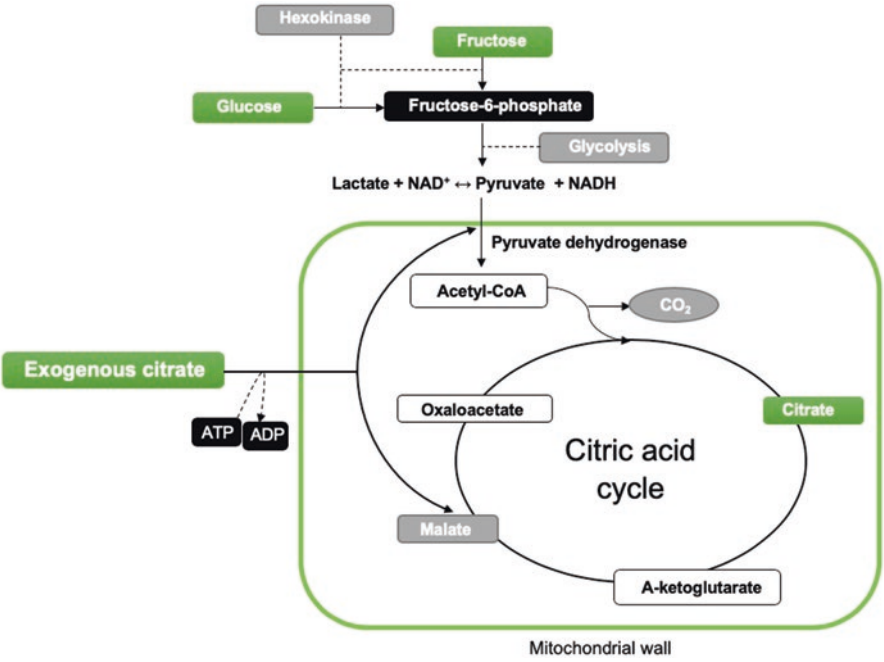

Fig. 3 Fructose and glucose enter the glycolytic pathway after phosphorylation by hexokinase and are subsequently converted to pyruvate via glycolysis to be used in the citric acid cycle. The entire citric acid cycle occurs within the inner mitochondrial wall. After entering the mitochondria through specific transporters, pyruvate can be oxidised to acetyl-coenzyme A (CoA) and CO_2. Acetyl-CoA then enters the citric acid cycle, which generates one high-energy bond in the form of GTP and four reduced compounds (three NADH and one FADH2) to be used in the oxidative phosphorylation process. Regarding citrate, when it is obtained from oxaloacetate inside the mitochondria as part of the citric acid cycle, exogenous citrate cannot permeate the inner mitochondrial membrane. Thus, before entering the cycle, it is first converted to malate and pyruvate by a series of enzymes using ATP. The electrons conserved in NADH and FADH2 are then used to reduce oxygen in the oxidative phosphorylation process. (Adapted from Visconti 2012 and Goss et al. 2019)

human (Cao et al. 2003) semen. With HSPA6 being upregulated after 4 h of EA, we may be observing an intrinsic response to epididymal storage of sperm from either the epididymal epithelial cells or the stored spermatozoa. Increased expression of heat shock proteins has been linked to ROS-related cell damage in sperm (Erata et al. 2008) and in other tissues (Dimauro et al. 2016; Mustafi et al. 2009; Zhang et al. 2018). Therefore, the increase in HSPA6 after 4 h of EA, may be due to the previous ejaculation, where levels of ROS-related sperm damage have been found to be higher than in periods of less than 1 day of EA (Agarwal et al. 2016; Gosálvez et al. 2011). This protection from ROS-related sperm damage may be contributing to the improved motility, which was seen after 4 h of EA.

To conclude, elucidating the mechanisms behind the improvement of sperm motility after EA periods of 4 h is clinically highly relevant in that a successive

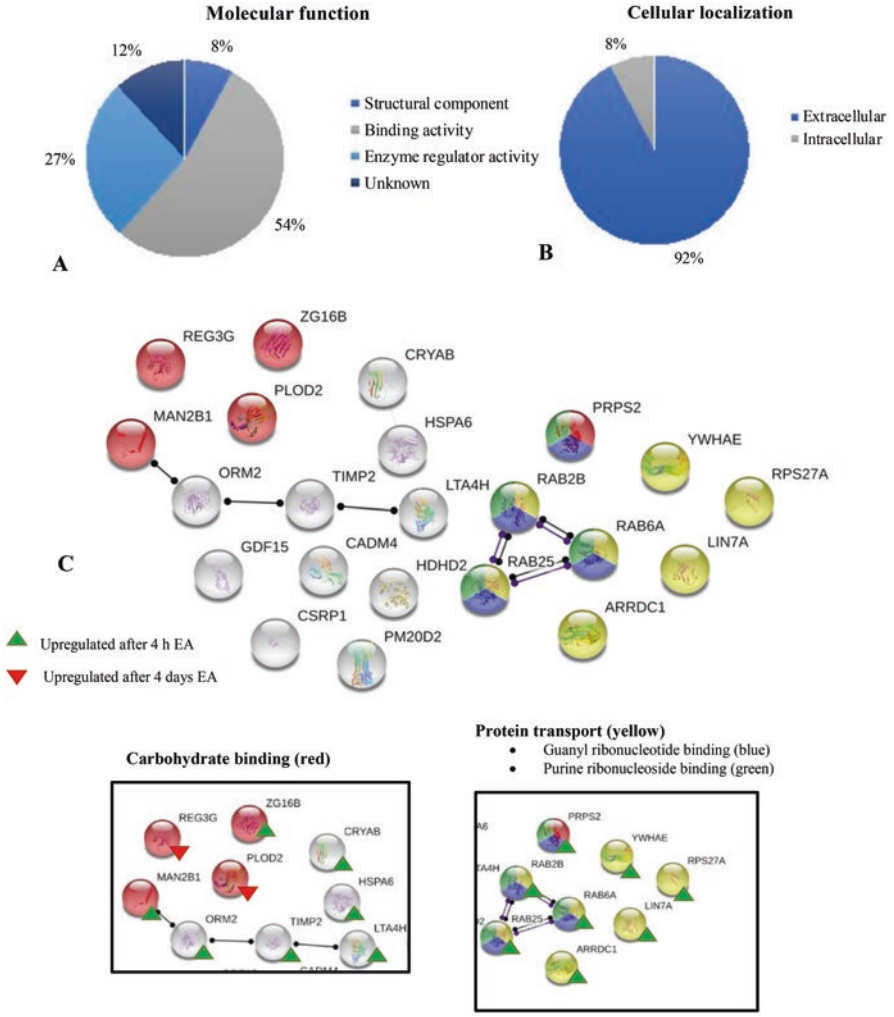

Fig. 4 GoMiner ontology analysis of molecular function (**a**) and cellular localization (**b**), protein interactions and clusters (**c**) revealed by STRING.org protein interaction database, of the 22 extracellular, differentially expressed proteins

ejaculation is often recommended in ART clinics after a poor sample is collected from a patient. Exploring the biochemical and physiological bases on which this practice is built upon, may serve to highlight the dynamic nature of sperm functional parameters in cases where EA is actively utilized and manipulated. This study highlights a gap in the understanding of how the seminal plasma microenvironment can impose notable changes on sperm motility and how the concentrations of the three major biomarkers of epididymal, prostatic and seminal vesicles secretion all decreased significantly after 4 h of EA, indicating reduction in the output of these

accessory sex glands, or a complex sperm selection mechanism relying on the physical positioning of sperm in the epididymis prior to ejaculation. A concomitant system of extracellular protein interactions has also been identified, giving an insight into the complex nature of this system and how many factors may influence sperm motility once sperm are ejaculated after variable EA.

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