Received: 28 May 2019 | Revised: 29 August 2019 | Accepted: 12 September 2019

DOI: 10.1111/and.13443

ORIGINAL ARTICLE

First International Journal of Andrology
ANDROLOGIA
WILEY

Possible factors influencing post-ejaculatory changes of the osmolality of human semen in vitro

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Funding information
Nidacon International AB

Abstract

The human ejaculate is made up of secretions from the different accessory sex glands that empty in sequence at ejaculation. However, the different secretions only mix completely in vitro when the entire ejaculate is collected in a container and handled in the laboratory. At ejaculation, proteins from the seminal vesicles form a gel in the ejaculate and semen cannot be properly analysed and processed until the gel is liquefied. During and after liquefaction, there is continuous enzymatic activities and an ongoing increase in osmolality. The aim of this study was to investigate possible factors that influence the increase in semen osmolality in vitro. Osmolality was measured by freezing-point depression. Prostatic secretion was measured as zinc concentration. The presence of spermatozoa neither influenced the actual measurement of semen osmolality, nor the increase in osmolality. Enzymatic inhibitors reduced the increase in osmolality, and semen dilution prevented any increase in semen osmolality. However, the increase in osmolality covaried with the seminal zinc concentration, indicating that the observed increase was related to factors of prostatic origin. A simple and convenient procedure to reduce the risk for osmotic challenges for spermatozoa during handling for assisted reproductive technologies might be early dilution of semen.

KEYWORDS
human ejaculate, prostate secretion, semen dilution, semen osmolality, sperm concentration

1 | INTRODUCTION

A substantial increase in extracellular osmolality will most likely disturb normal cell function. In contrast to blood and blood plasma, the ejaculate is not an entity within the body and lacks a control system for osmolality. Each ejaculate has an individual composition, formed at ejaculation by sequential expulsion of spermatozoa and the fluids from different auxiliary sex glands. In vivo, most of the spermatozoa are expelled early in the ejaculate, together with mainly isotonic prostatic fluid (Cooper, Barfield, & Yeung, 2005; Holmes, Björndahl, & Kvist, 2019; Lundquist, 1949; MacLeod & Hotchkiss, 1942), allowing the spermatozoa to quickly enter the isotonic cervical mucus (Rossato, Di Virgilio, & Foresta, 1996) without any substantial contact with seminal vesicular fluid (Björndahl & Kvist, 2003; MacLeod & Gold, 1951). This is in contrast with the situation in vitro, where spermatozoa are exposed to seminal vesicular fluid during and after liquefaction. This introduces a dual challenge in vitro: first cellular adjustment to increasing osmolality in the liquefied ejaculate, followed by exposure to a relatively low osmolality when exposed to an isotonic sperm selection medium.

During liquefaction, the functional characteristics of the ejaculate change. The gel-forming semenogelins originating from the seminal vesicles are transformed, resulting in a liquefied seminal fluid (Lilja, Abrahamsson, & Lundwall, 1989; Mann & Lutwak-Mann, 1981). There is pronounced enzymatic activity during and after liquefaction (Lilja & Weiber, 1984; Mann, 1964), which results in a rapid increment in choline and free amino acids in the seminal fluid (Lundquist, 1952; Mann, 1964), the choline being formed by dephosphorylation

of phosphorylcholine secreted from the seminal vesicles. The enzyme cleaving phosphorylcholine is acid phosphatase which is present in the prostatic fluid (Lundquist, 1947). Free amino acids are formed by proteolysis of the seminal vesicular proteins by enzymes of prostatic origin (Lundquist, 1952).

Semen osmolality increases during and after liquefaction (Abraham-Peskir, Chantler, Uggerhoj, & Fedder, 2002; Chantler & Abraham-Peskir, 2004; Cooper et al., 2005; Holmes et al., 2019; Rothschild, 1960). This osmolality increase is temperature dependent and is blocked by denaturizing temperature (Holmes et al., 2019). Therefore, it is likely that the osmolality increase is dependent on enzymatic degradation of molecules in semen (Kubicek, Lindner, & Santavy, 1959; Mann, 1964; Quinlivan, 1972). Furthermore, the degree of increase varies greatly between samples (Holmes et al., 2019). Taking into consideration the possible detrimental effects of osmotic challenges to cell functions, it was of interest to investigate possible factors influencing the development of semen osmolality in vitro. The duration of 3 or more hours of incubation was chosen as an experimental model to uncover intrinsic properties of human semen, and not to represent actual clinical procedures. To further elucidate the role of the enzymatic degradation, the effect of adding enzyme inibitors was also studied.

2 | MATERIALS AND METHODS

2.1 | Recruitment of ejaculates and basic assessments

Anonymous semen samples from the routine andrology laboratory were used. Ethical approval was obtained (Dnr2015/2326-31) for using the remaining patient semen samples after clinical assessments. All samples used were collected at the clinic by masturbation and investigated according to the World Health Organisation (WHO) recommendations (World Health Organization, 2010) and as specified by the Special Interest Group in Andrology of the European Society of Human Reproduction and Embryology (ESHRE-SIGA; Barratt, Björndahl, Menkveld, & Mortimer, 2011; Björndahl et al., 2010).

Results from analysis of the marker for the contribution of prostatic fluid (zinc), the marker for the contribution of seminal vesicular fluid (fructose) and the marker for the contribution of epididymal fluid (neutral α-Glucosidase activity) were obtained from routine analysis results. Zinc and fructose concentrations and alpha-glucosidase activity were measured in sperm-free seminal fluid (centrifugation 3,000 *g*, 20 min). Zinc was measured with a colorimetric assay (Wako Pure Chemicals GmbH; Johnsen & Eliasson, 1987). Fructose was determined using an acid indole assay (Karvonen & Malm, 1955). α-Glucosidase was assessed using an assay for the determination of neutral α-Glucosidase activity by measurement of the conversion of p-nitrophenol-glucopyranoside into p-nitrophenol which, together with Na_2CO_3, forms a complex that absorbs light of a certain wavelength making quantitation of the enzyme activity possible (Cooper, Yeung, Nashan, Jockenhovel, & Nieschlag, 1990). Total amounts of zinc ≥2.5 µmol/ejaculate (zinc concentration ≥1.2 mmol/ml), fructose ≥13 µmol/ejaculate and α-Glucosidase activity ≥20 mIU/ejaculate are considered indicative of normal prostatic, seminal vesicular and epididymal secretory functions, respectively (Björndahl et al., 2010).

All experimental incubations were done in capped test tubes at 37°C to avoid excessive evaporation.

2.2 | Osmolality assessment

The osmolality measurements were made on an automatic osmometer (Svenska Labex AB) utilising the principle of freeze point depression.

2.3 | Robustness of method for osmolality assessment

2.3.1 | Effect of sperm concentration

To evaluate the effect of the presence of spermatozoa on measurement of osmolality, sperm concentration was altered by serial dilution with seminal fluid from the same samples. Semen samples (n = 3) from men from infertile couples and with a semen volume above 3.0 ml and expected sperm concentration 50–100 × 10^6/ml were identified. The sample volumes were 3.4, 4.0 and 3.4 ml, respectively. Sperm concentration was assessed in the original sample (Björndahl et al., 2010; World Health Organization, 2010) and was 70, 78, 50 × 10^6/ml, respectively. After the initial semen examination, the samples were centrifuged at 400 *g* for 10 min, 2,100 µl of the supernatants was withdrawn and the sperm pellets resuspended in the remaining seminal fluid. Each sperm solution was then diluted in series by taking 400 µl from sperm solutions and adding 400 µl seminal fluid. From each native semen sample, five sperm suspensions were made, having 100%, 50%, 25%, 12.5% and 6.25% of the original sperm concentration and a sixth preparation containing the remaining sperm-free seminal fluid. Osmolality was then measured in the five sperm suspensions and in the sperm-free seminal fluid.

2.3.2 | Effect of filtration of semen and seminal fluid

To evaluate whether osmolality measurements were influenced by other particles present in the ejaculate, particles were removed by filtration.

1. Whole semen samples (WS; N = 3) were divided into two aliquots within 4 hr after ejaculation. Aliquot one acted as a control and aliquot two was filtered through a 0.2 µm acrodisc filter.
2. Seminal fluid (SF; without spermatozoa; N = 8) was prepared by centrifugation (3,000 *g*, 20 min) and divided into two aliquots within 2–4 hr after ejaculation. Aliquot one acted as control and aliquot two was filtered through a 0.2 µm acrodisc filter.

2.4 | Possible factors influencing semen osmolality increase

2.4.1 | Presence of spermatozoa

The potential role of spermatozoa for the increase in osmolality was studied in ejaculates of five men. Each sample was divided into two parts at 2 hr after collection. One part from each sample was subdivided into six aliquots (for three duplicate assessments) and remained as untreated WS. The other part from each sample was centrifuged 20 min at 3,000 *g*, and the sperm-free supernatant was then retrieved and similarly subdivided into six aliquots of SF.

Osmolality was assessed in duplicate of WS and SF, respectively, 2 hr after collection. The remaining aliquots were sealed and further incubated at 37°C. Osmolality was measured in duplicate 5 hr after ejaculation and the last aliquots 24 hr after ejaculation. This duration of incubation was used as an experimental model to study inherent properties of human semen and does not indicate a clinically relevant duration.

2.4.2 | Factors leaking from sperm prior to ejaculation

To evaluate whether enzymes and particles that may have leaked from spermatozoa significantly contribute to the high osmolality observed after liquefaction, osmolalities of ejaculates without spermatozoa were compared with the results from ejaculates with spermatozoa. The former data were obtained from azoospermic ejaculates and ejaculates from vasectomized men, and the latter from ejaculates with spermatozoa from men in infertile couples.

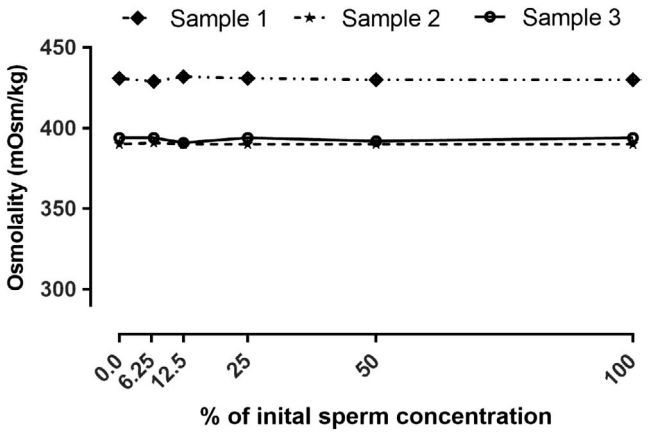

FIGURE 1 Osmolality (mOsm/kg) in three semen samples serially diluted from the initial concentration (100%) to 50%, 25%, 12.5% and 6.25%, respectively, of initial concentration. Zero value represents osmolality assessed in sperm-free seminal fluid. No difference in osmolality between different dilutions (one-way ANOVA: $p = .87$; n.s.; $df = 5$)

Osmolality was assessed directly after thawing of seminal fluid that had been frozen (−20°C) after centrifugation (3,000 *g*) 3–4 hr after ejaculation.

Azoospermic ejaculates

In total, 48 ejaculates from men referred for semen analysis due to unspecified couple infertility, where microscopic analysis of 20 µl semen did not reveal any spermatozoa, these were categorised as azoospermic.

Post-vasectomy ejaculates

In total, 40 semen samples from men referred to the clinic for control after a vasectomy, where semen analysis did not reveal any spermatozoa, these were categorised as sperm-free post-vasectomy samples.

Ejaculates containing spermatozoa

In total, 47 ejaculates containing spermatozoa were obtained from men referred for semen analysis due to unspecified couple infertility, 3–4 hr after ejaculation.

2.4.3 | Factors of epididymal, seminal vesicular and prostatic origin

Osmolality was measured in 47 semen samples from men referred for semen analysis due to unspecified couple infertility after liquefaction, at 25–60 min after ejaculation, and again 3 hr later after incubation at 37°C. This was compared with markers for prostatic, seminal vesicular and epididymal secretions, respectively.

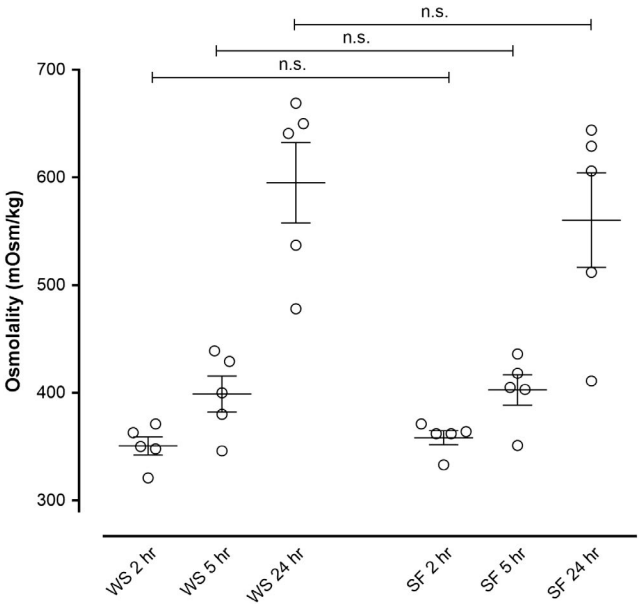

FIGURE 2 Development of osmolality (mOsm/kg) over time in whole semen (WS; presence of spermatozoa) and seminal fluid (SF; absence of spermatozoa) stored at 37°C to investigate the effect of presence of spermatozoa. Osmolality measured at 2, 5 and 24 hr after sample collection. Scatter plot with median, upper quartile and lower quartile (WS vs. SF: 2 hr, $p = .25$, n.s.; 5 hr, $p = .625$, n.s.; 24 hr, $p = .0625$, n.s.; Wilcoxon test; $N = 5$)

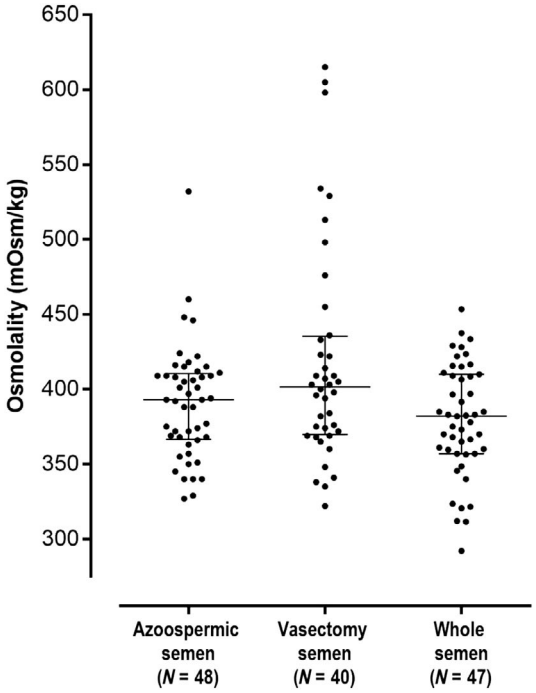

FIGURE 3 Osmolality (mOsm/kg) of semen without spermatozoa (azoospermic semen from men referred for unspecified couple infertility, *N* = 48 and semen from men tested after vasectomy, *N* = 40) and semen with spermatozoa (whole semen from men referred for unspecified couple infertility; *N* = 47). Absence of sperm did not reduce the increase in osmolality, slightly higher mean values for azoospermic and post-vasectomy ejaculates (393 and 401 vs. 382 mOsm/kg; one-way ANOVA; $p < .01$; scatter plot, median and interquartile range)

2.4.4 | Enzymatic activity

To evaluate whether enzymatic activity influences the increment in osmolality, seminal fluid was incubated at 37°C for 24 hr with and without a mixture of enzymatic inhibitors. Three stock solutions of inhibitors were made: (a) nuclease- and metallo-protease inhibitor EDTA (0.2 M Na_2-EDTA at neutral pH), (b) acid phosphatase inhibitor tartrate (1 M Na_2-tartrate) and (c) protease inhibitor mixture cOmplete™ (EDTA-free Protease Inhibitor Cocktail Roche; 1 tablet dissolved in 200 µl water 50×). Of each stock solution, 25 µl was added to 2.5 ml seminal fluid to give final concentrations EDTA 2 mM; tartrate 10 mM and cOmplete 0.5×. Aliquots of seminal fluid, frozen at 3–4 hr after ejaculation, were thawed, pooled and subdivided into two parts of 2.5 ml each. To the control, 75 µl water was added, and to the other part, 25 µl of each of the three inhibitor solutions was added. After addition of water or inhibitors, samples were thoroughly mixed. Osmolality was assessed in duplicates directly after mixing and again after 18 and 24 hr of incubation in a water bath at 37°C.

2.4.5 | Dilution of semen

To study how semen dilution might reduce the increment in semen osmolality, seven semen samples were diluted. From each of the

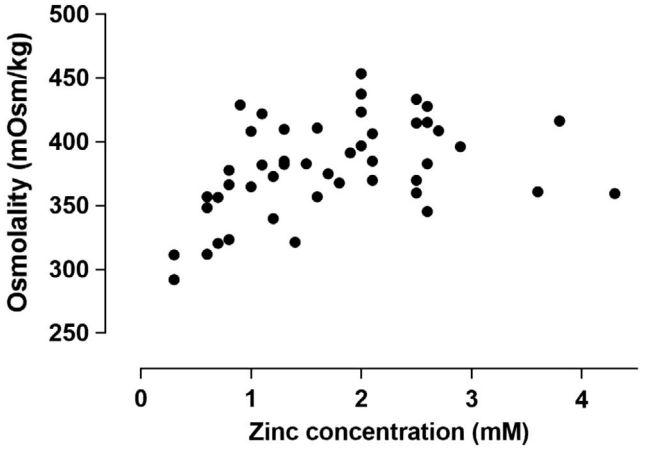

FIGURE 4 Relation between seminal zinc concentration (mmol/L) and osmolality (mOsm/kg) after 3 hr of incubation at 37°C ($r_c = .44$, $r^2 = .19$, $p < .01$; $N = 47$)

semen samples, two aliquots of 400 µl were withdrawn after complete liquefaction. To aliquot one, an equal amount of Earle's balanced salt solution with 10% human serum albumin (EBSSA) was added. Aliquot two remained undiluted. Osmolality assessments were done in undiluted aliquots and diluted aliquots, before and after 3 hr of incubation at 37°C.

2.5 | Statistical methods

Wilcoxon matched-pairs signed-rank test, Pearson correlation test and one-way ANOVA were performed using GraphPad Prism 7.04 for Windows, GraphPad Software, "www.graphpad.com." A *p*-value of <.05 was considered significant.

3 | RESULTS

3.1 | Robustness of method for osmolality assessment

Serial dilution of spermatozoa with sperm-free seminal plasma from the same sample did not change the measured osmolality in three ejaculates (one-way ANOVA: $p = .87$; n.s.; $df = 5$; Figure 1).

Filtration of WS (*N* = 3) or seminal plasma (*N* = 8) through 0.2 µm filter did not change the measured osmolality. Unfiltered WS showed a mean osmolality of 358 mOsm/kg, and filtered semen had 359 mOsm/kg (*N* = 3; $p = .75$, n.s. Wilcoxon test). Unfiltered seminal fluid showed a mean of 385 mOsm/kg and filtered seminal fluid a mean of 384 mOsm/kg (*N* = 8; $p = .58$, n.s. Wilcoxon test).

3.2 | Possible factors influencing semen osmolality increase

3.2.1 | Presence of spermatozoa

There was no relation between the sperm concentration and the osmolality after 3 hr of incubation at 37°C ($r_c = .10$, $p = .50$;

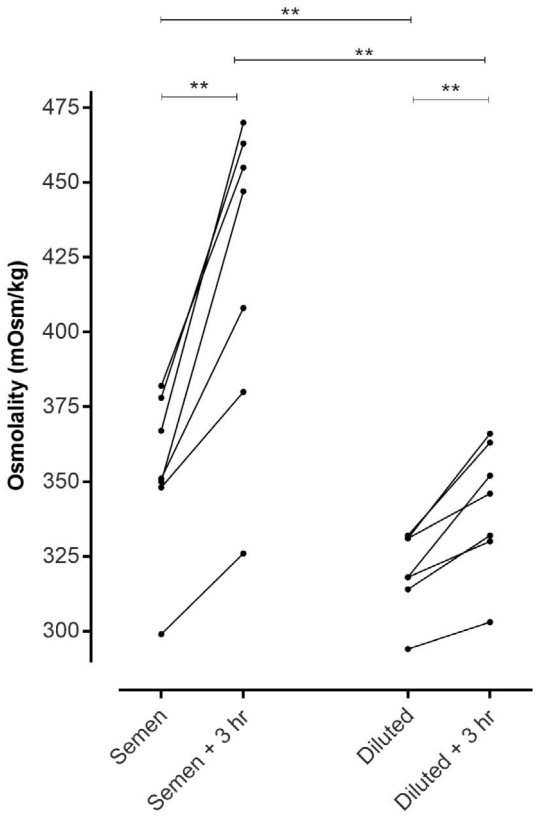

FIGURE 5 Osmolality (mOsm/kg) and osmolality development in whole semen and whole semen diluted with Earle's medium before and after 3 hr incubation at 37°C (N = 7). Increase in diluted semen was significantly lower (p < .01, Wilcoxon test) than in undiluted semen

$N_1 = N_2 = 47$). Furthermore, semen osmolality increased to similar levels in WS and sperm-free seminal plasma over time (N = 5, WS vs. SF: 2 hr, p = .25, n.s.; 5 hr, p = .625, n.s.; 24 hr, p = .0625, n.s.; Wilcoxon test; Figure 2).

3.2.2 | Factors leaking from sperm prior to ejaculation

The mean osmolality was high in all groups (Figure 3). Semen without spermatozoa (azoospermic and post-vasectomy) did not have significantly lower osmolality 3–4 hr after ejaculation when compared to ejaculates with spermatozoa (Figure 3). Azoospermic and post-vasectomy ejaculates had slightly higher mean semen osmolality (393 and 401, respectively, vs. 382 mOsm/kg, one-way ANOVA; p < .01).

3.2.3 | Factors of epididymal, seminal vesicular and prostatic origin

Osmolality measured after liquefaction at 25–60 min after ejaculation showed a high covariation with osmolality after 3 hr of incubation (r_c = .93, p < .0001, $N_1 = N_2 = 47$, r^2 = .86). The concentration of zinc was positively related to the osmolality 3 hr after liquefaction (r_c = .44, r^2 = .19, p < .01; N = 47; Figure 4). Thus, 19% of the variation in osmolality covaried with the zinc concentration. In contrast, there was neither a relation 3 hr after ejaculation between osmolality and fructose concentration (r_c = .11; p = .5, N = 47; n.s.), nor between osmolality and alpha-glucosidase activity (r_c = .16, p = .3; N = 47; n.s.).

3.2.4 | Enzymatic activity

In seminal fluid incubated with a mixture of inhibitors, the increment in osmolality was reduced by 83% (mean of duplicate measurements; from 133 to 22 mOsm/kg) after 18 hr of incubation and by 75% (from 170 to 42 mOsm/kg) after 24 hr of incubation.

3.2.5 | Dilution of semen

Dilution reduced the initial osmolality from on average 354 (range 299–382) to 320 mOsm/kg (294–332; N = 7; p < .01; Wilcoxon test; Figure 5: Semen vs. Diluted). In undiluted semen, osmolality increased during 3 hr of incubation by on average 68 mOsm/kg (27–103; N_1 = 7, p < .01, Wilcoxon test; Figure 5: Semen vs. Semen +3 hr), while in diluted semen, the increase was only 22 mOsm/kg (9–35; N_2 = 7, p < .01, Wilcoxon test; Figure 5: Diluted vs. Diluted +3 hr). The difference in increase was significant ($N_1 = N_2$ = 7, p < .01, Wilcoxon test).

4 | DISCUSSION

4.1 | Robustness of method for osmolality assessment

The measurement itself was not influenced by the presence of spermatozoa or other large particles and cells (e.g., blood cells, epithelial cells, bacteria and debris), since neither serial dilution of semen with autologous seminal fluid (Figure 1), nor centrifugation (Figure 2, 2 hr) or filtration changed the results of osmolality assessments.

4.2 | Possible factors influencing semen osmolality increase

The inherent property of osmolality increase was evident already soon after liquefaction: 86% of the variation in osmolality at 25–60 min after ejaculation covaried with the variation in osmolality after 3 hr of incubation.

The increase in osmolality over time did not depend on factors from spermatozoa, since exclusion by centrifugation did not change the increment (Figure 2). Further support was that, there was no relation between the osmolality increase and sperm concentration in 47 samples.

Factors potentially leaking from spermatozoa prior to ejaculation did not seem to contribute significantly to the observed increment in osmolality. Complete absence of spermatozoa before ejaculation (azoospermic and post-vasectomy semen) did not result in a lower increment in semen osmolality compared with samples from men

from infertile couples (Figure 3). Factors of epididymal origin did not appear to influence the increase in osmolality, since absence of epididymal factors (post-vasectomy semen) did not result in a lower increase in osmolality (Figure 3). Further support was that there was no relation between the increment and the marker for epididymal fluid. In addition, factors of seminal vesicular origin did not appear to be of importance for the rate of increase in osmolality, since there was no relation between the increment and the marker for seminal vesicular fluid.

Factors of prostatic origin appear to have contributed to the increase in osmolality. There was a significant relation between osmolality after 3 hr of incubation and the concentration of the prostatic marker zinc (Figure 4). The prostatic secretion is the main source of enzymes that can cleave proteins and other seminal vesicular compounds (Kubicek et al., 1959; Lilja & Weiber, 1984; Lundquist, 1947, 1949, 1952; Mann, 1964; Mann & Lutwak-Mann, 1981; Quinlivan, 1972).

A likely mechanism for the increase in semen osmolality would be enzymatic cleavage of molecules generating more osmotically active particles. In support of this was that addition of enzyme inhibitors significantly reduced the increment in osmolality. Further support for this is that the semen osmolality increment is temperature dependent and abolished by denaturizing temperature (Holmes et al., 2019).

Changes in pH were not part of the present experiments. However, the pH is likely to differ between different samples, depending on the relation between prostatic (pH 6.6–7.0) and seminal vesicular fluid (slightly alkaline). Since the increase in osmolality was related to the contribution of prostatic fluid to the ejaculate it is possible that the samples with the highest increase in osmolality also had more acidic prostatic fluid and, therefore, a lower pH initially.

Proteolysis in semen can be counteracted by dilution (Bucht, Arver, & Sjoberg, 1986). In the present study, the increase in osmolality was counteracted by diluting the sample with a buffered salt solution.

In conclusion, the large increase in human semen osmolality during and after liquefaction varied significantly between different semen samples and seemed to be dependent on factors inherent to each individual sample. The observed increase in semen osmolality appeared to be dependent on enzymatic processes and related to prostatic factors (Figure 4). Semen dilution prevented most of the osmolality increase (Figure 5). A simple and convenient procedure to reduce the risk for a dual osmotic challenge for spermatozoa during handling for ART, therefore, could be early dilution of the semen. Further studies should investigate to what extent osmotic challenges during in vitro handling can influence functional properties of human spermatozoa.

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How to cite this article: Holmes E, Björndahl L, Kvist U. Possible factors influencing post-ejaculatory changes of the osmolality of human semen in vitro. *Andrologia*. 2019;51:e13443. https://doi.org/10.1111/and.13443