Seminal Plasma and IVF Potential

Biochemical Constituents of Seminal Plasma of Males from In Vitro Fertilization Couples¹

MELISSA F. LAY,² M. ELAINE RICHARDSON,^{2,4} WILLIAM R. BOONE,³ ASHBY B. BODINE,² and RONALD J. THURSTON²

Submitted: January 10, 2000

Accepted: September 20, 2000

Purpose: Many components of seminal plasma play a role in sperm motility by serving as energy sources. Human seminal plasma contains over 30 proteins, including forward motility proteins, antifertility proteins, and coagulation/liquefaction proteins. This study was designed to determine any correlation between motility or fertilization rates and concentrations of fructose, lactic acid, citric acid, carnitine, and protein in human seminal plasma.

Methods: Fertilization rates were determined by in vitro methods. Fructose, lactic acid, citric acid, and carnitine concentrations were ascertained using high performance liquid chromatography. Protein concentration was determined by Bradford assay.

Results: Protein concentrations were significantly different as a function of sperm motility levels. Other constituents of human seminal plasma showed an overall correlation, though not significant. No constituent exhibited significant differences as a function of fertility levels.

Conclusions: Protein concentration was significantly lower for samples with high motility. No significant differences between fertility levels and constituents measured were found.

KEY WORDS: Amines; carbohydrates; in vitro fertilization; semen; seminal plasma.

INTRODUCTION

In the past, female infertility was believed to be the predominant causative factor influencing a couple's inability to conceive. In the 1970s, researchers began to relate male infertility with procreation problems. Now it is believed that 10–20% of all couples are infertile, and in 30% of these couples, the male is implicated as the primary cause of the infertility. Techniques such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) allow these infertile couples to increase their chances of conceiving (1). The establishment that male infertility can be a predominant problem renewed interest in semen analysis as a diagnostic technique in the assessment of infertility.

Throughout the male reproductive tract, accessory sex glands and the epididymis produce seminal plasma that contains the compounds required for spermatozoan metabolism, motility, and fertilization as well as for coagulation and liquefaction. Seminal plasma proteins are secreted from all of the accessory sex glands. A human ejaculate contains 3–5% protein (2–5). The major serum/seminal plasma proteins have been identified as prealbumin, albumin, globulins, mucin, nucleoprotein, α and β immunoglobulins, and transferrin (4,6,7).

The primary energy source for spermatozoal motility comes from the breakdown of fructose (5). Fructose constitutes approximately 25% of the total dry weight (315 mg/100 ml) of the seminal vesicle secretions in man (2,4–6,8). Davis and McCune (9) positively correlated fructose utilization with sperm concentration in human semen. They reported

¹ Research was funded by SCAES Project #1513.

² Animal and Veterinary Sciences Department, Clemson University, Clemson, South Carolina.

³ Reproductive Endocrinology and Infertility, Greenville Hospital System, Greenville, South Carolina.

⁴ To whom correspondence should be addressed at Clemson University, Animal and Veterinary Sciences Department, Box 340361, 125 Poole Ag Center, Clemson, South Carolina 29634-0361.

that high-motility specimens utilized fructose at a rate two times greater than did low-motility specimens. As fructose is transformed anaerobically, there is a linear increase in lactic acid concentrations (5,10). Unlike other mammalian cells, spermatozoa possess an intramitochondrial lactate dehydrogenase, necessary to utilize lactate as a mitochondrial substrate (11,12).

Carnitine is found in many different tissues, including the epididymis (13); it is accumulated and concentrated from the blood by epithelial cells in the caput and corpus epididymis (14). From these areas, carnitine is secreted into the lumen and accumulates in the cauda region of the epididymis. It functions as a shuttle for the energy source for spermatozoa for most domestic animals and humans (15). In the human, the mean epididymal concentration of total carnitine is 6 mM (2,14). In 1981, Hinton and co-workers (16) demonstrated that high carnitine concentrations significantly influenced motility of rat spermatozoa within the first 2 min of exposure. The initial stimulation of motility in the caput epididymis was then inhibited by the high concentrations and prolonged exposure to carnitine in the cauda epididymis (16,17). Upon ejaculation, carnitine is diluted by fluids from the remaining secondary sex glands, allowing the initiation of motility via metabolic processes of the spermatozoa.

Human prostatic fluid makes up 15–30% of an ejaculate; citric acid is the major component ranging from 100 to 700 mg/100 ml (2,4). The level of citric acid in an ejaculate reflects the androgen levels of that mammal and the pathology of the prostate (5). Hamamah (18) concluded that considerable interindividual variations occur in citrate levels. Kavanagh (19) found that the concentration of citrate was positively correlated with Ca^{2+} and K^{+} . In most mammals, citrate maintains osmotic equilibrium of the major cations in seminal plasma (5,19,20).

Several studies for predictors of IVF rates have been reported recently in which the activity of several seminal plasma enzymes was evaluated. Rolf (21) determined that creatine kinase activity cannot be used as a predictor of male fertility in an IVF program. Similarly, Spiessens and co-workers (22) were unable to successfully predict pregnancy outcomes of participants in an IVF program by evaluating α -glycosidase activity. The present study was designed to quantify fructose, citric acid, lactic acid, carnitine, and protein in human seminal plasma and to evaluate their relationship to fertilization potential of semen specimens in an IVF program.

MATERIALS AND METHODS

Patients

Twenty-four females with a mean ($\pm$ SD) age of 32.7 ± 4.7 years participated in this study. Primary infertility diagnoses for these women included tubal factor (46%), endometriosis (27%), ovulatory dysfunction (12%), male factor (12%), and pelvic adhesive disease (4%). The male partners had a mean age ($\pm$ SD) of 33.9 ± 4.8 years, a mean semen volume of 3.4 ± 1.4 ml, a mean sperm concentration of 79.4 ± 72.2 million/ml, a mean sperm motility of $50.2 \pm 24.1\%$, and a mean normal sperm morphology by 1992 WHO standards (23) of $23.5 \pm 12.4\%$.

Semen Preparation for In Vitro Fertilization (IVF)

The evaluation of semen parameters began at Greenville Hospital System. Specimens were obtained from the male partner of 26 infertile couples who, in 1994–1995, had chosen IVF as a means to conceive. All samples were collected by masturbation and placed in an incubator at 37°C within 30 min of ejaculation. Within 1 h of liquefaction, volume, pH, sperm motility, sperm concentration, sperm agglutination, and osmolality of seminal plasma were assessed (23). The percentage of morphologically normal sperm was determined at a later date. Once the above parameters were recorded, 5 ml of a simple salt solution containing HEPES and human serum albumin called sperm wash medium (SWM) (Irvine ScientificTM, Santa Ana, CA) were added to the total volume of semen. The mixture of semen and SWM was centrifuged at $300 \times g$ for 5 min, forming a sperm pellet and a diluted seminal plasma supernatant. The pellet was overlaid with a simple salt solution containing human serum albumin (Human Tubal Fluid; Irvine Scientific, Santa Ana, CA). The sperm were allowed to swim for an hour up into the salt solution with the supernatant being removed and used for IVF. The mean in vitro-fertilization rate for this study group was 57% (160 zygotes/282 oocytes). The supernatant, containing only seminal plasma and SWM, was decanted, frozen in 1-ml aliquots in liquid nitrogen, transported to Clemson University and stored in liquid nitrogen until assays were performed.

Preparation of Seminal Plasma for Protein Determination

The 1-ml aliquots of seminal plasma samples containing SWM were thawed in an ice bath and

quantitatively transferred to a Centricon-10 (10 kDa nMWCo) centrifuge tube (Amicon7, Beverly, MA). Before centrifugation, 10 μ l of benzamidine (Sigma, St. Louis, MO), a serine protease inhibitor, were added to the 1-ml volume of sample. The samples then were centrifuged at $5000 \times g$ for 1 h to separate the seminal plasma proteins from the lower MW compounds, that is, fructose, lactic acid, citric acid, and carnitine. One ml of 1% (w/v) glycine was added, and the centrifugation repeated. Each centrifugation was carried out for 1 h or until the 10 kDa nMW membrane was almost dry. The filtrate was collected, capped and frozen at -20°C for further use, whereas the 10 kDa nMWCo membrane was washed with 1-ml of 1% glycine. From the membrane wash, 200 μ l of sample were removed and diluted 1:5 with 1% glycine for total protein determination. The remaining 800 μ l were transferred to a Centricon-50 centrifuge tube containing a 50 kDa nMWCo membrane. Centrifugation at $2500 \times g$ of the Centricon-50 for 30 min allowed a separation of those proteins <50 kDa nMW and >50 kDa nMW. From both fractions, 200 μ l were assayed for total protein along with the previous sample from the Centricon-10.

Total protein concentrations were determined using a Bio-Rad Protein Assay (24) modified for a microplate reader. Absorbance was measured at 1570 nm using a Thermomax Microtiter Reader (Molecular Devices Corporation, Menlo, CA, USA) and 96-well microtiter plates. Seminal plasma protein levels were adjusted to reflect the addition of the protein in the SWM (5.75 mg/ml). All data were accumulated on the SoftMax Software package for Macintosh version 2.01 (81990 Molecular Devices Corporation, Menlo, CA, USA).

HPLC Analysis of Fructose from Human Seminal Plasma

Fructose concentrations were determined using high-performance liquid chromatography (HPLC). The Centricon-10 filtrate was thawed, 100 ml removed and diluted 1:20 with HPLC grade water for a final volume of 2 ml. The entire contents of the diluted sample were processed on a Dionex HPLC system. The HPLC system utilized a Dionex CarboPac PA-100 column (4×250 mm) and guard column, a Dx-300 standard advanced gradient pump module, and a Dionex autosampler (Dionex Corporation, Sunnyvale, CA, USA). All eluents were helium sparged in an Eluent Degas Module (Dionex Corporation, Sunnyvale, CA, USA).

Fructose was eluted over a 17-min period, using a ternary elution procedure. Eluent 1 consisted of 100% HPLC grade water. Eluent 2 was 0.2 N NaOH and eluent 3 was 0.5 M NaOAc in 0.2 N NaOH. The gradient method began with 100% of eluent 2 up to 13 min. At 13 min, 32% of eluent 3 and 68% of eluent 2 were pumped through the column. At 14 min, 100% of eluent 2 was pumped through the column until the end of the cycle. Detection of the samples was via refractive index, using a Pulsed Amperometric Detector (Dionex Corporation, Sunnyvale, CA, USA). Integration and data storage were performed with Dionex Chromatography software.

Analysis of Total Carnitine from Human Seminal Plasma

HPLC was performed using a Rainin Dynamax Gradient HPLC System (Rainin Instrumentation, Woburn, MA). The flow rate was 0.5 ml/min using an isocratic 80% methanol eluent. Samples were diluted 1:30 with HPLC grade water. Run time was 18 min with a retention time of 2.5 min. A 50 ml volume of the sample was resolved on a 5 m- C_{18} reverse phase column ($250 \text{ mm} \times 4.6 \text{ mm}$) (Alltech Associates, Deerfield, IL, USA) at room temperature. All eluents were prefiltered and degassed through a 0.45-mm membrane. External and internal standards of L-carnitine ranging between 0.25 and 1.0 mg/ml (Sigma, St. Louis, MO, USA) were used for quantitation. Accumulation and integration of the data were performed on a MacClassic 20sc System, using Dynamax Image software (Apple Computer, Cupertino, CA, USA) and printed on a Macintosh Image Writer II. Carnitine was detected at 1215 nm/0.05 AUFS, using a Kratos Spectroflow 757 UV/Vis detector.

Dual Detection of Lactic Acid and Citric Acid

Lactic acid and citric acid were separated on an organic acid IOA-1000 column ($300 \text{ mm} \times 7.8 \text{ mm}$) and guard column (Alltech Associates, Deerfield, IL, USA), using a Rainin Dynamax HPLC System. Pre-filtered and degassed 0.005 N sulfuric acid eluent was pumped through the system at a flow rate of 0.5 ml/min. Lactic acid eluted at 16.8 min and citric acid at 10.3 min in a 20-min cycle. Standards of L-lactic acid (Sigma, St. Louis, MO, USA) and citric acid (Sigma, St. Louis, MO, USA), ranging between 0.25 and 1.0 mg/ml, were processed on a 20-min elution period. All samples were diluted 1:10 in HPLC grade

water. Detection of the separated compound was performed using a Spectroflow 757 absorbance detector at 1210 nm/0.05 AFUS. Integration of the data was performed by the Dynamax Image software package.

Statistical Analysis

All data were analyzed using the SAS program Pearson's correlation coefficient (SAS, Cary, NC, USA). The analysis of human seminal plasma constituents with fertility levels was by Analysis of Variance and Tukey's Studentized range distribution. Comparative analysis of the motility levels with the human seminal plasma constituents was by SAS *t*-test (SAS, Cary, NC).

RESULTS

A total of 24 male patients consented to further semen analysis outside the normal sperm parameter techniques. There was a negative correlation between the concentrations of fructose, citric acid, and carnitine and the percent of oocytes fertilized in vitro (Table I). Percent motility displayed a negative correlation with fructose, lactic acid, protein, carnitine, and citric acid, but not to a significant level ($P > .05$), Table I.

The concentrations of citric acid, lactic acid, carnitine, and protein decreased as motility increased. However, only the decrease in protein concentration ($46.84 \pm 6.79\%$ vs. $30.11 \pm 3.74\%$) was statistically significant ($P < .05$), Table II. When percent fertility was divided into three arbitrary groups consisting of low fertility ($\leq 30\%$), medium fertility (31–70%) and high fertility ($\geq 71\%$), no significant differences ($P > .05$) were observed among fertility

Table I. Correlation of Fertility and Motility with Human Seminal Plasma Constituents of Males ($n = 24$) from In Vitro Fertilization Couples

Seminal plasma constituent	Fertility	Motility
Fructose	–0.0898 0.6765	0.4045 –0.1783
Lactic acid	0.2134 0.3168	–0.2690 0.2037
Citric acid	–0.1168 0.5867	–0.2473 0.2441
Carnitine	–0.0453 0.8332	–0.2687 0.2042
Protein	0.0209 0.9228	–0.3415 0.1025

Note. First row entries indicate Pearson's correlation coefficient; the second row entries indicate probability.

Table II. Average Values of Seminal Plasma Constituents in the Semen of Males with Different Sperm Motility Indicators^a

Seminal plasma constituent	Low motility ^b ($n = 13$)	High motility ^c ($n = 11$)
Fructose	4.49 ± 0.69^d	5.06 ± 1.11^d
Lactic acid	17.17 ± 2.48^d	14.58 ± 2.10^d
Citric acid	16.15 ± 1.58^d	14.59 ± 2.82^d
Carnitine	67.50 ± 8.12^d	53.48 ± 9.57^d
Protein	46.84 ± 6.79^d	30.11 ± 3.74^e

Note. Values represent mean $\pm$ SE.

^a Values expressed as mg/ml of seminal plasma.

^b $\leq 50\%$.

^c $\geq 51\%$.

^{d,e} Numbers within rows with different superscripts differ ($P < .05$).

levels and the human seminal plasma constituents of fructose, citric acid, lactic acid, carnitine, and protein (Table III). Pregnancy rates were not listed because some transferred embryos were a result of ICSI, whereas others were a result of donor spermatozoa.

DISCUSSION

This study was designed to determine whether a correlation exists between motility and/or fertility of spermatozoa and the seminal plasma constituents fructose, lactic acid, citric acid, carnitine, and protein. A total of 24 samples were obtained from infertile couples who elected in vitro fertilization as an alternate technique for conception.

Fructose

Biswas (25) established a significant negative correlation between fructose concentration and sperm motility from patients attending an infertility clinic. We also determined a negative correlation between

Table III. Average Values of Seminal Plasma Constituents in the Semen of Males with Varying In Vitro Fertilization Efficiencies^a

Seminal plasma constituent	Low fertility ^b ($n = 5$)	Medium fertility ^c ($n = 8$)	High fertility ^d ($n = 11$)
Fructose	4.23 ± 0.463	4.88 ± 0.289	4.02 ± 0.211
Lactic acid	11.48 ± 1.716	19.13 ± 1.073	16.11 ± 0.780
Citric acid	16.71 ± 1.604	13.28 ± 1.003	15.01 ± 0.729
Carnitine	52.62 ± 6.777	66.46 ± 4.235	59.72 ± 3.080
Protein	33.73 ± 3.681	47.55 ± 2.761	36.19 ± 1.841

Note. Values represent mean $\pm$ SE.

^a Values expressed as mg/ml of seminal plasma.

^b $\leq 30\%$.

^c ≥ 31 –70%.

^d $\geq 71\%$.

fructose and motility, though not statistically significant. The populations termed by Biswas and co-workers (25) as infertile or fertile were based on classification of the male ejaculate as either azoospermic or oligozoospermic. The classification of our study population was based on IVF percentages. Possibly, if the data reported by Biswas and co-workers (25) were reclassified as low and high fertility or fertile and infertile groups as in the present study, and if we had increased the population size of our study, the results would be in closer agreement.

Sakkas *et al.* (26) examined the effects of fructose on IVF of mouse embryos and concluded that fructose supported fertilization, but not cleavage. In a study by Jeyendran and colleagues (27), no relationship was found between fertilization and fructose levels although a lower concentration of fructose in the infertile group was observed. In no case was a correlation between fructose and fertility sufficient to estimate whether a semen sample was fertile. Although our study indicated a lower concentration of fructose in both the low-fertility and the high-fertility groups compared to the medium fertility group, the differences were not statistically significant supporting the results of Jeyendran and colleagues (27).

Lactic Acid and Citric Acid

Lactic acid and citric acid concentrations were determined by a method similar to Oefner and associates (28), using HPLC to elute citric acid, inositol and fructose in boar and bull seminal plasma. According to Storey and Kayne (12), lactate, the end product of fructolysis, as well as other catabolic processes, can be utilized directly as an energy source. The movement of lactic acid via the lactate/pyruvate shuttle may cause an increase in motility. Therefore, one could expect lactic acid concentrations to be lower in samples with high motility; and, in our study, a negative relationship was determined between motility and lactic acid concentrations.

Citric acid concentrations in our study did not show a significant relationship with motility or fertility levels. Kavanagh (19) stated that citric acid in the TCA cycle contributes little energy to mitochondrial oxidative phosphorylation. Citric acid plays more of a role in transport of ions and osmotic balance of semen. Calcium uptake is important for sperm motility via cAMP and protein phosphorylation. Citric acid levels in our study (10–17 mg/ml) were an order of magnitude higher than values reported by other researchers (2,4). The couples in our study have been unable

to conceive without assisted reproductive techniques and are, therefore, not considered a normal population. It is then possible that the increased concentration of citric acid in our study was affected by the source of the specimens. Further investigation is warranted.

Carnitine

Mature spermatozoa contain high concentrations of endogenous carnitine and acetylcarnitine upon ejaculation, as well as high carnitine acetyltransferase activity (29). Our study observed a mean total carnitine concentration of 60.49 mg/ml. Other researchers observed total carnitine values ranging from 0.2 to 1.3 mM (0.03–0.21 mg/ml) (2,30,31). We measured carnitine by HPLC, using a C₁₈ column and absorption at 215 nm. Earlier researchers determined carnitine through enzymatic calorimetric assay (31,32) or gas chromatography (33). If one assumes that the precision of HPLC compared to enzymatic assays is much greater, this could account for the increased values found in our study. A distinction between free L-carnitine and acetylated carnitine was not made in our study. Therefore, the concentrations of carnitine measured could be additive due to acylcarnitine derivatives. Acylcarnitine derivatives make up 50% of total semen carnitine (31,34). However, the carnitine concentration of our study remained higher than that reported by previous researchers even when taking into account the measurement of acetylcarnitine.

The correlation between carnitine and motility or fertility was negative, but not statistically significant. The carnitine in seminal plasma could be taken into the spermatozoa and utilized. This could be more as a buffering system for acetyl-CoA produced in the mitochondria (35). Levels of free L-carnitine in seminal plasma are the same for fertile and infertile men (34). It is the levels of acetylcarnitine that are significantly lower in oligospermic and azoospermic human semen. A difference between fertility levels and total carnitine was not observed. However, our classification of fertile and infertile samples was based on IVF rates, not reduced spermatozoa numbers as in the study by Kohengkul (34).

Proteins

Human seminal plasma contains serine proteases that hydrolyze other proteins. The process of semen coagulation/liquefaction is affected by these proteases. To inhibit the serine proteases, 10 μ l of 1 mM

benzamidine were added per ml of diluted seminal plasma. Benzamidine also was added to the 1% glycine wash medium used during separation of the proteins.

The mean seminal plasma protein concentration (30.1 mg/ml) in our study was in agreement with values reported by other researchers (2,3) (18–50 mg/ml). However, Mann (5) observed protein concentrations between 350 mg/ml and 550 mg/ml. This difference between researchers could be due to the sensitivity of the Bio-Rad Protein Assay compared to the colorimetric assay utilized by Mann (5).

Bovine seminal plasma (BSP) contains four acidic proteins, BSPA₁, A₂, A₃, and BSP-30. These proteins are involved in acrosomal reactions and binding of the sperm to the oocyte (36–39). These BSP proteins coat the sperm membrane, altering its permeability. The BSP proteins are considered bovine fertility proteins. Human seminal plasma contains proteins with molecular weights similar to the BSP proteins, but their functions are unknown. There were no significant differences observed between protein concentrations and fertility levels in this study; however, the couples of the present study were from a population of people who could not conceive naturally.

When compared with motility levels, protein concentration exhibited a statistically significant difference. Rodent epididymal fluid contains proteins which initiate motility of epididymal sperm (40,41). One of these proteins is similar to albumin with a MW of approximately 66 kDa. Lindholmer (42) showed that human sperm motility increases with increased concentrations of albumin. The sperm wash medium used in our study contains 5.75 mg/ml of protein, predominantly albumin (95–99% pure albumin), which possibly could have affected the motility of the samples used.

CONCLUSIONS

The results of our research have not revealed significant statistical differences between fertility levels and the human seminal plasma constituents fructose, citric acid, lactic acid, carnitine, and protein. However, there was a negative correlation observed between mean fructose value and mean fertility that is in agreement with previous research. Protein concentration was significantly lower for samples with high motility, but the remaining constituents did not differ significantly. The overall correlation of the constituents

with motility exhibited the same negative correlation as protein; however, these correlations were not statistically significant.

In conclusion, these data confirm the findings of others and help to establish the concentration of fructose, lactic acid, and protein in seminal fluid in man. However, our increased values for carnitine and citric acid may represent more accurate values for these organic compounds, or these values may be specific for males experiencing infertility. If the latter is true, evaluating semen specimens for these compounds prior to expensive IVF procedures may be relevant. It may be that the addition of these compounds into culture media prior to IVF will increase fertilization rates and ultimately pregnancy rates. Further investigation into these two compounds is warranted and should include comparisons of a larger number of fertile as well as infertile individuals. In addition, day-to-day variability should also be investigated in both groups to determine if major fluctuations occur. If major fluctuations do occur, this line of investigation would need to be replaced with biochemical markers of spermatozoa.

REFERENCES

1. Yablonsky T: Male Fertility Testing. *Lab Med* 1996;27(6):378–383
2. Setchell BP, Brooks DE: Anatomy, vasculature, innervation and fluids of the male reproductive tract. *In* The Physiology of Reproduction, 2nd Ed, E Knobil, JD Neill (eds), New York, Raven Press, 1994, p 814
3. Ayyagari RR, Fazleabas AT, Dawood MY: Seminal plasma proteins of fertile and infertile men analyzed by two-dimensional electrophoresis. *Am J Obst Gyn* 1987;157(6):1528–1533
4. Mann T, Lutwak-Mann C: Male Reproductive Function and Semen. New York, Springer-Verlag, Berlin, 1981
5. Mann T: The Biochemistry of Semen and of the Male Reproductive Tract. New York, John Wiley and Sons, 1964
6. Zaneveld LJD, Chatterton RT: Biochemistry of Mammalian Reproduction. New York, John Wiley and Sons, 1982
7. Quinlivan WLG: Analysis of the proteins in human seminal plasma. *Arch Biochem Biophys* 1968;127:680–687
8. Tauber PF, Zaneveld LJD, Propping D, Schumacher GFB: Components of human split ejaculates. I. Spermatozoa, fructose, immunoglobulins, albumin, lactoferrin, transferrin and other plasma proteins. *J Reprod Fert* 1975;43:249–267
9. Davis ME, McCune WW: Fructolysis of human spermatozoa. *Fert Ster* 1950;1(4):362–372
10. Dhanotiya RS, Srivastava RK: Relationship between sperm concentration, initial fructose concentration and different measures of fructolysis in buffalo semen. *J Agr Sci in Cambridge* 1979;92:545–547
11. Calvin J, Tubbs PK: Mitochondrial transport processes and oxidation of NADH by hypotonically-treated boar spermatozoa. *European J Biochem* 1978;89:315–320

12. Storey BT, Kayne FJ: Energy metabolism of spermatozoa. VI. Direct intramitochondrial lactate oxidation by rabbit sperm mitochondria. *Bio Reprod* 1977;16:549–556
13. Pande SV, Parvin R: Carnitine–acylcarnitine translocase-mediated transport of fatty acids into mitochondria: Its involvement in the control of fatty acid oxidation in liver. *In* Carnitine Biosynthesis, Metabolism and Functions, RA Frenkel, JD McGary (eds), London, Academic Press, 1980, pp 143–155
14. Menchini-Fabris GF, Canale D, Izzo PL, Olivieri L, Bartelloni M: Free L-carnitine in human semen: Its variability in different andrological pathologies. *Fert Ster* 1984;42(2):263–267
15. Brooks DE: Carnitine in the male reproductive tract and its relation to the metabolism of the epididymis and spermatozoa. *In* Carnitine Biosynthesis, Metabolism, and Functions, RA Frankel, JD McGary (eds), London, Academic Press, 1980, pp 219–235
16. Hinton BT, Brooks DE, Dott HM, Setchell BP: Effects of carnitine and some related compounds on the motility of rat spermatozoa from caput epididymis. *J Reprod Fert* 1981;61:59–64
17. Hinton BT, Setchell BP: Concentration and uptake of carnitine in the rat epididymis: A micropuncture study. *In* Carnitine, Biosynthesis, Metabolism, and Functions, RA Frenkel, JD McGary (eds), London, Academic Press, 1980, pp 237–248
18. Hamamah S, Seguin F, Barthelemy C, Akoka S, LePape A, Lansac J, Royere D: H nuclear magnetic resonance studies of seminal plasma from fertile and infertile men. *J Reprod Fert* 1993;97:51–55
19. Kavanagh JP: Sodium, potassium, calcium, magnesium, zinc, citrate and chloride content of human prostatic and seminal fluid. *J Reprod Fert* 1985;75:35–41
20. Huggins C: The physiology of the prostate gland. *Phys Rev* 1945;25:281–293
21. Rolf C, Behre HM, Cooper TG, Koppers B, Nieschlag E: Creatine kinase activity in human spermatozoa and seminal plasma lacks predictive value for male fertility in in vitro fertilization. *Fert Ster* 1998;69(2):727–734
22. Spiessens C, D'Hooghe T, Wouters E, Meuleman C, Vanderschueren D: α -Glycosidase activity in seminal plasma: Predictive value for outcome in intrauterine insemination and in vitro fertilization. *Fert Ster* 1998;69(4):735–739
23. World Health Organization: WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 3rd Ed. Cambridge, Cambridge University Press, 1992
24. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochem* 1976;72:248–254
25. Biswas S, Ferguson KM, Stedronska J, Baffoe G, Mansfield MD, Kosbab MH: Fructose and hormone levels in semen: Their correlations with sperm counts and motility. *Fert Ster* 1978;30(2):200–204
26. Sakkas D, Urner F, Menezes Y, Leppens G: Effects of glucose and fructose on fertilization, cleavage and viability of mouse embryos in vitro. *Bio Reprod* 1993;49:1288–1292
27. Jeyendran RS, Van der Ven HH, Rosecrans R, Perez-Pelaez M, Al-Ha S, Zaneveld LJD: Chemical constituents of human seminal plasma: Relationship to fertility. *Andrologia* 1989;21(5):423–428
28. Oefner P, Bonn G, Bartsch G: High-performance liquid chromatographic analysis of citric acid, inositol and fructose in boar and bull seminal plasma. *Andrologia* 1985;17(3):250–253
29. Bremer J: Carnitine—metabolism and functions. *Phys Rev* 1983;63(4):1420–1465
30. Suter DA, Holland MK: The concentrations of free L-carnitine and L-O-acetylcarnitine in spermatozoa and seminal plasma of normal, fresh, and frozen human semen. *Fert Ster* 1979;31:541–544
31. Soufir JC, Ducot B, Marson J, Jouannet P, Feneux D, Soumah A, Spira A: Levels of seminal free L (–) carnitine in fertile and infertile men. *Int J Androl* 1984;7:188–197
32. Brooks DE: Carnitine, acetylcarnitine and the activity of carnitine acyltransferases in seminal plasma and spermatozoa of men, rams and rats. *J Reprod Fert* 1979;56:667–673
33. Lewin LM, Beer R, Lunenfeld B: Epididymis and seminal vesicle as sources of carnitine in human seminal fluid: The clinical significance of the carnitine concentration in human seminal fluid. *Fert Ster* 1976;27(1):9–13
34. Kohengkul S, Tanphaichitr V, Muangmun V, Tanphaichitr N: Levels of L-carnitine and L-O-acetylcarnitine in normal and infertile human semen: A lower level of L-O acetylcarnitine in infertile semen. *Fert Ster* 1977;28(12):1333–1336
35. Casillas ER, Erickson BJ: The role of carnitine in spermatozoan metabolism: Substrate-induced elevations in the acetylation state of carnitine and Coenzyme A in bovine and monkey semen. *Biol Reprod* 1975;12:275–283
36. Desnoyers L, Therien I, Manjunath P: Characterization of the major proteins of bovine seminal fluid by two-dimensional polyacrylamide gel electrophoresis. *Mol Reprod Dev* 1994;37:425–435
37. Manjunath P, Chandonnet L, Leblond E, Desnoyers L: Major proteins of bovine seminal vesicles bind to spermatozoa. *Bio Reprod* 1993;49:27–37
38. Desnoyers L, Manjunath P: Major proteins of bovine seminal plasma exhibit novel interactions with phospholipid. *J Biol Chem* 1992;267(14):10149–10155
39. Manjunath P, Sairam MR: Purification and biochemical characterization of three major acidic proteins (BSP-A₁, BSP-A₂, BSP-A₃) from bovine seminal plasma. *Biochem J* 1987;241:685–692
40. Brooks DE, Higgins SJ: Characterization and androgen-dependence of proteins associated with luminal fluid and spermatozoa in the rat epididymis. *J Reprod Fert* 1980;59:363–375
41. Cameo MS, Blaquier JA: Androgen-controlled specific proteins in rat epididymis. *J Endo* 1976;69:47–55
42. Lindholmer C, Carlstrom A, Eliasson R: Occurrence and origin of proteins in human seminal plasma with special reference to albumin. *Andrologia* 1974;6(2):181–196