The impact of L-carnitine on human semen quality upon cryopreservation, its association with sperm fatty acids, and assessment of a novel analytical tool for DNA fragmentation in mammals

Mario Iliceto

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Faculty of Health Sciences

OsloMet – Oslo Metropolitan University

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Abstract

Reduced male fertility is associated with reduced semen quality and can result from medical conditions, such as infections or blockages in the reproductive organs, and lifestyle factors like obesity. However, the cause of reduced fertility remains unexplained in about 50% of affected males. Even though semen analysis constitutes a central and initial part of male infertility investigation, relying on it alone is insufficient for a comprehensive diagnosis of male infertility. Recent studies suggest that oxidative stress may be a contributing factor in male infertility. Oxidative stress arises from an imbalance between reactive oxygen species (ROS) production and the body's antioxidant defence, leading to sperm DNA damage at various stages of sperm development. While sperm fatty acids are vital for sperm function, L-carnitine is equally important, facilitating the transport of long-chain fatty acids for β-oxidation and supporting antioxidant defence. High levels of L-carnitine from the epididymis are found in seminal plasma and positively correlate with sperm concentration and motility. Additionally, L-carnitine supplementation in cryopreservation media improves post-thaw semen quality by reducing ROS levels and DNA fragmentation.

The primary objective of this project was to assess the effect of endogenous seminal L-carnitine on human sperm parameters in fresh and post-thaw semen samples. Additionally, we explored alternative approaches for evaluating DNA fragmentation, using bull spermatozoa as a model system. In the first article, we revealed that free seminal L-carnitine levels positively correlate with palmitic acid, docosahexaenoic acid, and total n-3 PUFAs in sperm. Seminal L-carnitine levels were not linked to BMI, indicating that the reduced semen quality associated with obesity is unrelated to L-carnitine. These results support the role of the epididymis in sperm maturation.

In the second paper, we showed that both endogenous L-carnitine and L-carnitine supplemented to the cryopreservation medium improve sperm motility and mitigate the oxidative stress caused by freezing. In addition, this study evaluated alternative methods to the Sperm Chromatin Structure Assay (SCSA) for detecting DNA damage in bovine sperm. Our study found that SCSA and Sperm Chromatin Dispersion (SCD) tests both effectively identified increased DNA damage in bull spermatozoa exposed to high H_2O_2 concentrations, whereas Sensitive Recognition of Individual DNA Ends (dSTRIDE) test did not. These results suggest that SCD is a promising alternative to SCSA for assessing DNA damage, while dSTRIDE needs further refinement.

Sammendrag

Redusert mannlig fertilitet er forbundet med nedsatt sædkvalitet og kan ha ulike årsaker som medisinske tilstander, som infeksjoner eller blokkeringer i reproduktive organer, eller livsstilsfaktorer som fedme. Hos rundt 50% av menn med nedsatt fertilitet er årsaken fortsatt ukjent. Selv om en sædanalyse utgjør en sentral og innledende del av undersøkelsen av mannlig infertilitet, er den alene ikke tilstrekkelig for å stille en fullstendig diagnose. Nyere forskning antyder at oksidativt stress kan være en medvirkende faktor til mannlig infertilitet. Oksidativt stress oppstår som følge av en ubalanse mellom produksjon av reaktive oksygenforbindelser (ROF) og kroppens antioksidantforsvar, noe som kan føre til DNA-skader i sædceller i ulike stadier av spermieutviklingen. Fettsyrer er viktige for sædfunksjonen, mens L-karnitin spiller en sentral rolle ved å transportere langkjedede fettsyrer for β-oksidasjon og styrke antioksidantforsvaret. Høye nivåer av L-karnitin fra epididymis finnes i sædplasma og viser en positiv sammenheng med sædkonsentrasjon og motilitet. Tilsetning av L-karnitin i frysemedia har vist å forbedre kvaliteten på opptint sæd ved å redusere ROF-nivåer og DNA-skader.

Hovedmålet med dette prosjektet var å undersøke effekten av endogent seminalt L-karnitin på sædparametere i både ferske og tinte sædprøver hos mennesker. I tillegg ble alternative metoder for å vurdere DNA-fragmentering testet ved bruk av sædceller fra okse som dyremodell.

Vår studie viste en positiv sammenheng mellom endogene nivåer av L-karnitin i sædplasma og fettsyrer som palmitinsyre, docosahexaensyre og totale n-3 flerumettede fettsyrer. Det ble ikke funnet noen sammenheng mellom L-karnitin-nivåer og BMI, noe som antyder at redusert sædkvalitet assosiert med fedme er uavhengig av L-karnitin. Funnene våre bekrefter rollen til epididymis i spermiemodning. Studien viste også at både endogene nivåer og tilsatt L-karnitin forbedret sædmotilitet, reduserte oksidativt stress og beskyttet mot fryseindusert skade. Videre testet vi alternative metoder til Sperm Chromatin Structure Assay (SCSA) test for å oppdage DNA-skader i oksesæd. Resultatene viste at både SCSA og Sperm Chromatin Dispersion (SCD) tester effektivt identifiserte økt DNA-skade i prøver eksponert for høye H_2O_2-konsentrasjoner, mens Sensitive Recognition of Individual DNA Ends (dSTRIDE) test ikke gjorde det. Dette indikerer at SCD er et lovende alternativ til SCSA for å vurdere DNA-skader, mens dSTRIDE krever videre utvikling av metoden.

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Levels of L-carnitine in human seminal plasma are associated with sperm fatty acid composition

Authors: Mario Iliceto, Mette Haug Stensen, Jorunn M. Andersen, Trine B. Haugen, Oliwia Witczak

Asian Journal of Andrology 2024, 24 (5), 451-457, DOI: 10.4103/aja2021107

Paper II:

Association of endogenous seminal L-carnitine levels with post-thaw semen parameters in humans

Authors: Mario Iliceto, Mette Haug Stensen, Jorunn M. Andersen, Trine B. Haugen, Oliwia Witczak

Andrologia 2024, 24 (1), 4327010, DOI: 10.1155/2024/4327010

Paper III:

Assessment of alternative methods to Sperm Chromatin Structure Assay for evaluating DNA damage induced by oxidative stress in bull sperm

Authors: Ingvild Aas, Mario Iliceto, Else-Berit Stenseth, Anne Hege Alm-Kristiansen, Oliwia Witczak, and Erwan Delbarre

Manuscript

Abbreviations

ART Assisted reproductive technology

CACT Carnitine-acylcarnitine translocase

CPA Cryoprotective agent

CPT1 Carnitine palmitoyltransferase 1

CPT2 Carnitine palmitoyltransferase 2

CT2 Carnitine transporter 2

DFI DNA fragmentation index

DHA Docosahexaenoic acid

DS-DBs Double strand-DNA brakes

FSH Follicle-stimulating hormone

GnRH Gonadotropin-releasing hormone

H_2O_2 Hydrogen peroxide

ICSI Intracytoplasmic sperm injection

IVF In vitro fertilization

LH Luteinizing hormone

MUFAs Monounsaturated fatty acids

NADPH Nicotinamide adenine dinucleotide phosphate

$^1O^2$ Singlet oxygen

$O^{2\bullet-}$ Superoxide anion

OCTN2 Organic cation/carnitine transporter 2

$^{\bullet}OH$ Hydroxyl radical

PUFAs Polyunsaturated fatty acids

ROS Reactive oxygen species

SCD Sperm Chromatin Dispersion

SCSA Sperm Chromatin Structure Assay

SFAs Saturated fatty acids

SHBG Sex hormone-binding globulin

sORP Static oxidative-reductive potential

SS-DBs Single strand-DNA brakes

STRIDE Sensitive Recognition of Individual DNA Ends

WHO World Health Organization

1. Introduction

1.1 Background

Approximately 10-15% of couples of reproductive age struggle with fertility issues, and an increasing number of couples visit assisted reproduction clinics for infertility treatments. The causes of infertility are often attributed equally to female and male factors, each accounting approximately for about 40%, with the remaining 20% being unexplained [1, 2]. Reduced male fertility is linked to sperm concentration, morphology, and motility and can be attributed to several factors, including genetic and hormonal causes, medical conditions such as infections or blockages in the reproductive organs, as well as lifestyle factors like obesity [3]. However, in about 50% of males with reduced fertility, the etiology is still unexplained [4].

Semen analysis constitutes a central and initial part of male infertility investigation, where sperm parameters such as volume, sperm concentration, motility and morphology are assessed [5]. Among these, sperm motility is especially crucial, as it plays a key role in both natural and assisted conception processes [6]. Today, semen analysis is conducted in accordance with the World Health Organization (WHO) guidelines, providing standardized procedures and reference values [5, 7]. While semen analysis offers valuable insights, relying on it alone is insufficient for a comprehensive diagnosis of male infertility [8].

In fertility clinics, semen cryopreservation is a common practice for male fertility preservation, used for assisted reproduction [9], prior to radiation or chemotherapy [10], or for storing donor sperm [11]. However, cryopreservation has been found to negatively impact sperm motility and induce DNA damage [12-14]. Cryopreservation-induced damage can result from various mechanisms, including cold shock, osmotic stress, formation of intracellular ice crystals, and oxidative stress [15].

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defence systems [16]. Excessive ROS production can result in sperm DNA damage, which may occur at various stages of sperm development, ranging from spermatogenesis to ejaculation.

While polyunsaturated fatty acids (PUFAs), abundantly present in spermatozoa, significantly contribute to semen quality [17-19], the role of the amino acid derivative, L-carnitine, is also

crucial. L-carnitine not only aids in the transport of long-chain fatty acids to the mitochondria for β-oxidation [20], but it also contributes to antioxidant defence [21].

High levels of L-carnitine deriving from the epididymis, are detectable in seminal plasma and exhibit a positive relationship with key sperm parameters such as concentration and motility [22, 23]. Moreover, cryopreservation media supplemented with L-carnitine has demonstrated efficacy in mitigating damage inflicted by the freezing process on spermatozoa, resulting in enhanced semen quality after thawing [24, 25] along with a reduction in ROS levels and DNA fragmentation in spermatozoa [26].

Animal models have been instrumental in elucidating fundamental principles of male reproduction [27]. Among these models, larger animals like bovines offer a closer biological resemblance to humans [28, 29]. The use of bull spermatozoa in research is particularly advantageous owing to their ability to yield a high number of spermatozoa [30] for extensive experimentation. This has facilitated investigations into various aspects of sperm biology, including ROS and sperm DNA integrity contributing to the development of innovative approaches for preserving male fertility [31-33].

1.2 Male reproductive system

The male reproductive system includes the penis, testes, and duct system with accessory glands. The testicular region includes the testicle and the epididymis. The testicular parenchyma, which is enclosed by a fibrous membrane called the tunica albuginea, consists of seminiferous tubules, packed within testicular lobules, and interstitial tissue containing both a connective and a cellular component [34]. The tunica albuginea divides the testis into circa 250 lobules, each containing highly coiled seminiferous tubules (Figure 1). The seminiferous tubules are lined with a stratified epithelium composed of two types of cells: the germ cells and the Sertoli cells. Sertoli cells provide nutrients and hormones to germ cells and are organized to form tight junctions, creating a barrier between blood vessels and the testis called the blood-testis barrier [34]. This barrier is responsible for the maintenance of microenvironment which is suitable for germ cell development [34]. The interstitial tissue contains blood vessels and Leydig cells which are responsible for the endocrine function of the testicle by producing testosterone [34].

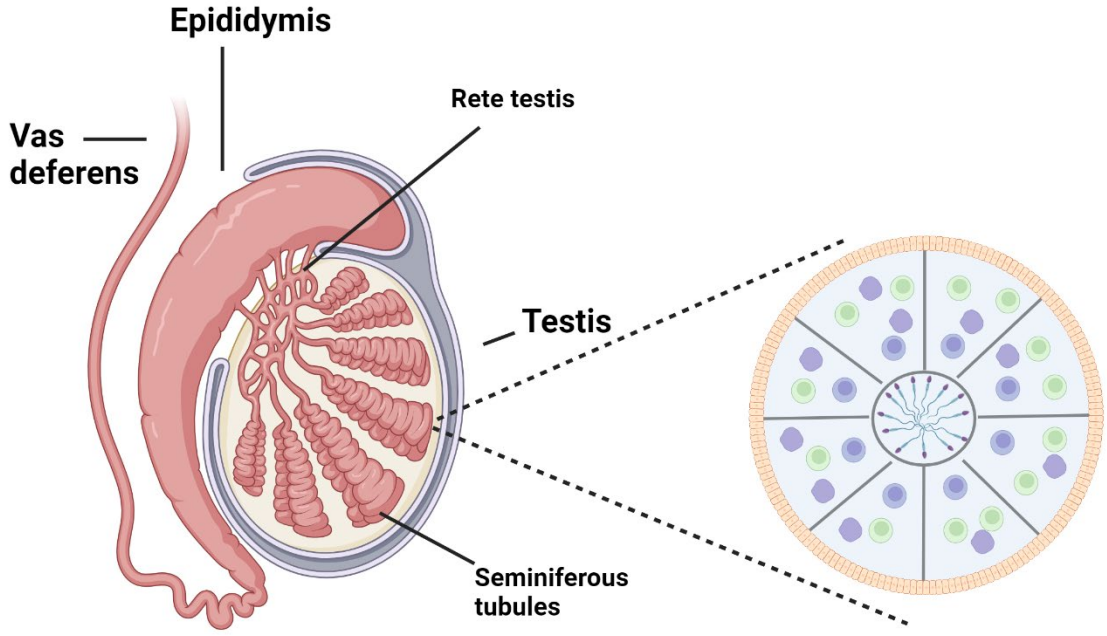

Figure 1. Illustration of the testis. *Cross-section of the testis and the epididymis, and a single seminiferous tubule. The seminiferous tubules within the testis are the site of spermatogenesis. The rete testis functions as a network of channels that transport sperm from the seminiferous tubules to the epididymis, where they are stored and matured. Illustration is made by the author using BioRender.com*

1.2.1 Spermatogenesis

Spermatogenesis is the process that leads to the formation of spermatozoa from spermatogonia (Figure 2) [34]. It begins from primordial germ cells, which are the precursors of both male and female gametes during the initial stages of embryogenesis [35]. The primordial germ cells migrate towards the gonad, colonizing it, and undergoing differentiation to give rise to the first male sex cells, the type-A spermatogonia [36]. Until the onset of puberty, these cells, localized within the sex cords, can undergo proliferation but not meiosis [37]. With the onset of puberty, type-A spermatogonia increase proliferation where one daughter cell becomes a new type-A spermatogonia and the other differentiates into a type B-spermatogonia which further differentiates becoming a primary spermatocyte [34]. The primary spermatocyte (diploid) will enter the first meiotic division to generate 2 secondary spermatocytes (haploid but still with both sister chromatids). With the second meiotic division, each of them will lead to the formation of 4 spermatids (haploid cells with single-chromatid chromosomes) [36]. The spermatids obtained will be the final precursors in the formation and maturation of sperm cells

through spermiogenesis [36], which involves, nuclear condensation, the formation of the flagellum, the development of the acrosomal cap and cytoplasmic reduction [36]. The mature spermatozoa are then transported to the epididymis for further maturation [38].

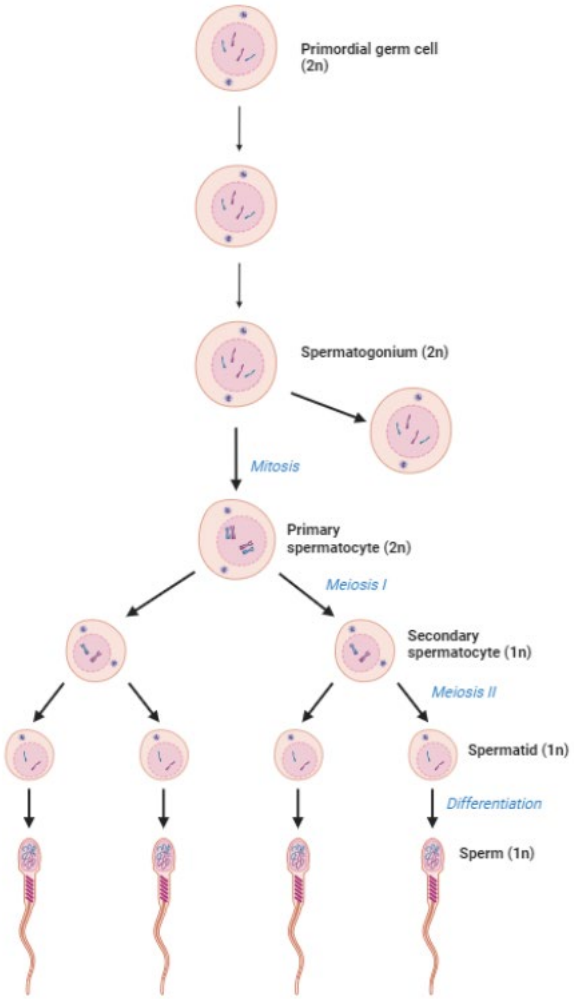

Figure 2. The process of spermatogenesis. *Spermatogenesis is the process through which primordial germ cells develop into mature spermatozoa. It involves mitotic divisions of spermatogonia, followed by two meiotic divisions of spermatocytes, resulting in haploid spermatids, which differentiate into spermatozoa. Illustration made by the author using BioRender.com*

1.2.2 Reproductive hormones

Spermatogenesis is regulated by a complex network of central and peripheral signals that respond to various internal and environmental factors. The neuroendocrine control of spermatogenesis involves three endocrine organs: the hypothalamus which produces and

releases gonadotropin-releasing hormone (GnRH); the anterior lobe of the pituitary gland (adenohypophysis), where gonadotropic cells, stimulated by GnRH, produce and release luteinizing hormone (LH) and follicle-stimulating hormone (FSH), known as gonadotropins; and the gonads, which, at beginning of puberty, generate gametes and respond to gonadotropins by increasing the synthesis and release of sex steroids and peptide hormones [34, 39]. LH triggers testosterone production in Leydig cells, while FSH, in conjunction with testosterone, activates Sertoli cells, which through a paracrine mechanism, oversee the maturation of gametes [40]. Sertoli cells also contain the enzyme aromatase which converts testosterone into the oestrogen estradiol. Oestrogens together with gonadal hormones regulate the secretion of GnRH and gonadotropins through a feedback mechanism on the hypothalamus and pituitary gland [34].

Testosterone and oestrogen are primarily transported through the bloodstream by SHBG (Sex Hormone Binding Globulin), a plasmatic glycoprotein [34]. Only a small portion of these hormones are unbound and free in the blood plasma, constituting their biologically active form [34]. The binding of testosterone and oestrogen to SHBG regulates the concentration of bioavailable steroids and influences the balance between bioavailable oestrogens and androgens [34, 41]. This balance is crucial for processes such as spermatogenesis.

Spermatogenesis could be negatively impacted by obesity [42]. The reduced number of spermatozoa in men with high BMI can be caused by a disturbance of the hypothalamic-pituitary-gonadal axis. Reduced serum and tissue testosterone levels result from the high conversion of androgens to oestrogens by aromatase, as the enzyme is also present in white adipose tissue [43]. On the other end, the increased oestrogen levels, combined with a reduced LH secretion, inhibit the hypothalamus-pituitary-gonadal axis through a feedback mechanism, further lowering the testosterone levels [43]. Reduced hepatic synthesis of SHBG can be observable in obese men [44]. This is due to a high-calorie diet rich in sugars, and higher insulin levels which are the most important factors in inhibiting SHBG synthesis which leads to lower circulating testosterone [45].

1.2.3 Epididymis and its main functions

The epididymis is a convoluted duct comprising the initial segment, the caput, the corpus, and the cauda, each with specialized functions [46]. However, in humans, while it displays the same segments, its proximal region is largely composed of efferent ducts (Figure 3) [47].

Figure 3. Human epididymis. *Dissected human epididymis tissue, highlighting the efferent duct (ED), caput (CAP), corpus (COR), and cauda (CAU) regions. Size bar = 1 cm. Figure retrieved from Leir and colleagues, 2020 [47] under the CC-BY 4.0 license and modified by the author.*

The epididymis is conserved across reptiles, avians, and mammals alike, granting the transport of the spermatozoa from the testicle to the vas deferens, a process typically taking 10–15 days [48]. This organ increases sperm concentration primarily through fluid absorption, where most of the fluid leaving the rete testis is absorbed [48, 49]. This absorption allows for a higher sperm concentration in the cauda epididymis compared to the rete testis [48, 50]. High sperm concentrations in the epididymis are then reflected in the ejaculate.

One of the main functions of the epididymis is sperm maturation which involves modification of the composition of sperm plasma membrane, chromatin condensation, and cytoskeletal reorganization [48, 51, 52]. Thanks to these modifications, spermatozoa in the cauda epididymis end up being fully mature and are able to display a progressive motility pattern, which is crucial in male fertility [48, 53].

Another function of the epididymis is to protect sperm cells from damage induced by extrinsic environmental factors [48, 54]. The blood-epididymis barrier also plays a crucial role in safeguarding maturing sperm cells from the immune system, thus providing an additional layer of protection [55]. It is also responsible for the unique composition of epididymal luminal fluid, which differs from that of blood plasma, with molecules such as carnitine being concentrated 100-fold [46]. Epididymal fluid contains an array of antioxidant enzymes such as superoxide dismutase glutathione peroxidase, glutathione-S-transferases and catalase, that play a role in decreasing the deleterious effects of ROS in spermatozoa [56]. ROS are chemically reactive molecules containing oxygen, such as superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and singlet oxygen ($^1O_2$), produced as by-products of cellular metabolism [57-59]. The epididymal fluid is also rich in organic solutes. Several studies identified organic solutes such as glutamate, taurine, myoinositol, glyceryl-phosphorylcholine, phosphorylcholine, sialic acids, amino acids and hormones distributed differently in the epididymal duct of many species, including the human [60, 61].

1.2.4 Seminal plasma

Seminal plasma fluid is a complex secretion originating from various glands within the male reproductive system. These include the seminal vesicles, prostate, epididymis, and bulbourethral (Cowper's) gland (Figure 4).

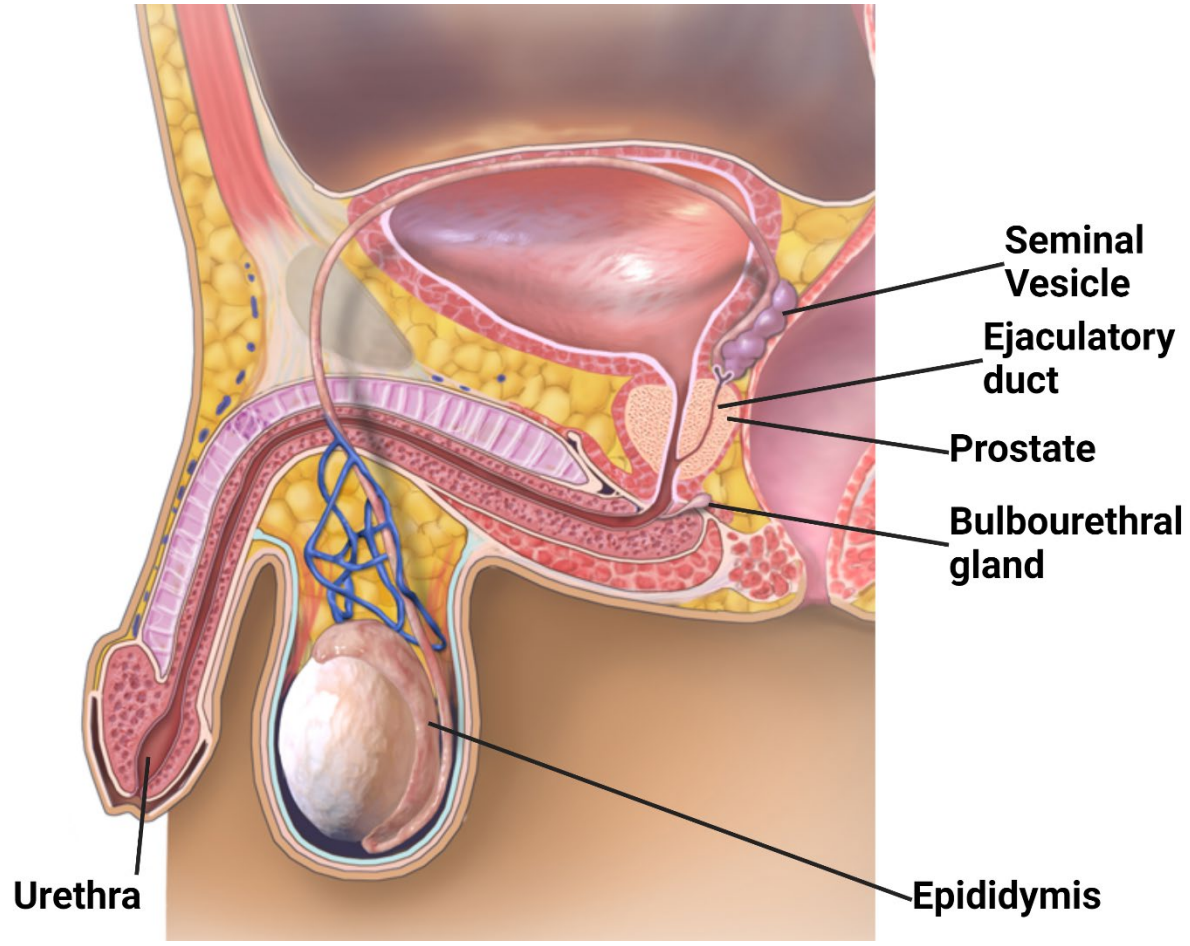

Figure 4. Cross-sectional view of the male reproductive system. *Sperm originating in the testes travels through various ducts before being released via the urethra. Along this journey, secretions from the accessory glands combine with the sperm to form the ejaculate (Image credit: "Varicocele" by BruceBlaus, under the CC BY-SA 4.0 license, modifications and labeling by the author).*

During ejaculation, spermatozoa traverse the ejaculatory ducts, merging with these glandular secretions to form semen [62]. Seminal plasma, the resultant fluid after the cellular component is removed, is derived principally from the seminal vesicle (65%) and prostate (25%) [63]. Seminal plasma fulfils multiple roles: it supports sperm nutrition, affects sperm behavior in the female reproductive tract by interacting with the immune system, and importantly regulates semen properties such as coagulation, liquefaction, sperm motility, and fertilization [62].

Seminal plasma consists of a wide array of molecules, including extracellular DNA, RNA, microRNAs, short aminoacidic sequences, proteins, short-chain carbohydrates, fatty acids, polysaccharides, inorganic ions, and small molecule metabolites such as carnitines [62, 64-68]. Lipids and fructose serve as the main energy sources for spermatozoa [62, 69, 70]. Zinc, a prevalent inorganic ion, assumes a multifaceted role as a cofactor or inhibitor for many proteolytic enzymes involved in the coagulation–liquefaction process [62, 71]. Copper and

selenium are key components of antioxidant enzyme systems and contribute significantly to normal spermatogenesis [62, 65].

The seminal vesicles contribute to the most significant molecular components of the seminal plasma [72, 73]. On the other hand, the prostate is characterized by a fluid rich in citrate, fatty acids, and proteolytic enzymes, to regulate sperm maturation, semen liquefaction, and sperm movement [73, 74]. Amines, such as spermine, spermidine, and putrescine, help sustain the alkaline nature of semen. This alkalinity, produced by vesicular and prostatic secretions, is essential for protecting sperm cells as they navigate the acidic conditions of the vaginal fluid [62, 75]. The secretion from the bulbourethral glands includes substances like galactose, sialic acid, and mucus. This secretion is involved in lubricating the semen, aiding in the smooth passage and effective transfer of sperm cells [76, 77].

1.2.5 Mature spermatozoa

A mature spermatozoon is composed of a head, mid-piece, and a tail (Figure 5) [78]. The head has a slightly flattened, pear-shaped structure that is about 4-5 µm long. It contains the nucleus, which appears as a dense mass of highly condensed chromatin with a haploid chromosome set, and the acrosome, a large lysosome packed with lytic enzymes such as hyaluronidase, acrosin and lipase [78]. Some of these enzymes are anchored to the inner side of the acrosomal membrane and are crucial during fertilization for breaking down the cumulus cells and zona pellucida. [78, 79]. The mid-piece is at most 1.5 times the length of the head [78] and contains mitochondria which provide the energy needed for cell motion [80]. A typical human sperm features 12-13 mitochondrial spirals, each containing a pair of mitochondria. A reduced function of mitochondria, which might be reflected in morphological abnormalities [14], is associated with reduced sperm motility [78, 81]. Approximately 60 µm long, the sperm tail (flagellum) features a central cylindrical structure called the axoneme encased in a fibrous sheath to form the axial filament complex [78]. The axoneme has a similar architecture to other cellular surface structures such as motile cilia and consists of a central pair of microtubules surrounded by nine peripheral doublets of microtubules [78]. Each doublet is composed of two subunits which facilitates the movement of the flagellum. In the midpiece, the axoneme is encased in nine dense outer fibers instead which are further surrounded by a mitochondrial sheath [78].

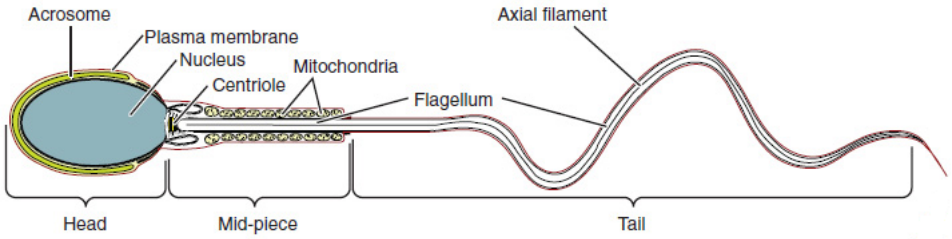

Figure 5: Schematic structure of a human spermatozoon. *A human spermatozoon consists of three main parts: the head, containing the nucleus and acrosome; the mid-piece, packed with mitochondria; and the tail (flagellum), which facilitates motility. (Image credit: "illustration from Anatomy & Physiology" by OpenStax College under the CC BY 4.0 license, modification by the author).*

1.3 Fatty acids

Fatty acids are fundamental components of lipids such as triacylglycerides and phospholipids (Figure 6). The latter are key elements of cell membranes, playing key roles in maintaining cell structure, facilitating function, storing energy, and being precursors for fat-soluble hormones [82]. Featuring a water-attracting head and two water-repelling fatty acid tails, phospholipids can self-organize into bilayer membranes in water [83].

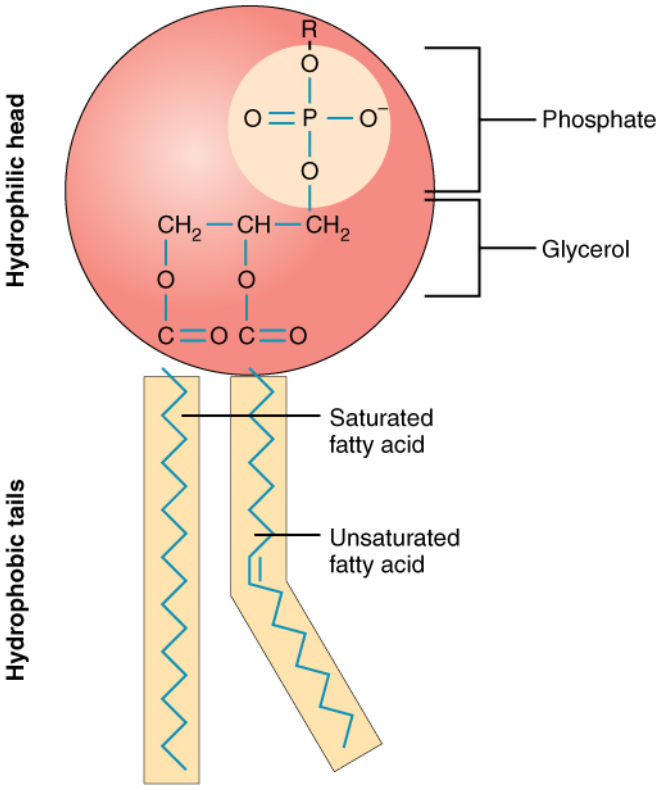

Figure 6. Structure of a phospholipid*. The diagram illustrates a polar head group along with two non-polar tails. The presence of double bonds causes a kink in one of the fatty acid tails. Image credit: "illustration from Anatomy & Physiology" by OpenStax College, under the CC BY 4.0 license.*

Fatty acids are hydrophobic molecules made up of a carboxyl group at one end, and a chain of carbon and hydrogen atoms ending with a methyl group at the other end [83]. The key structural differences between fatty acids are the presence and number of carbon-carbon double bonds, the length of the carbon chain, the positions of the double bonds, and the orientation of these double bonds (*cis* if on the same side of the double bond, creating a curved structure, and *trans* if on opposite sides, resulting in a straighter structure) [83].

In fatty acids, the carbon atoms are numbered starting from the carbon that contains the carboxyl group. The methyl carbon at the end of the chain is also called the ω (omega) carbon or n carbon. The position of double bonds can be expressed in two ways: the first method involves numbering the carbon atoms from the carboxyl end of the chain, with the position of the double bonds indicated by the Δ symbol followed by a superscript number; the second method involves indicating the position of a double bond using the ω or n classification, where the location of the first double bond is determined by counting from the end methyl group [83]. The type of bond between the carbon atoms determines whether the molecule is saturated or unsaturated.

Saturated fatty acids (SFAs) are characterized by their complete saturation with hydrogen atoms along their carbon chains, meaning there are no double bonds between carbon atoms (Figure 7). Monounsaturated fatty acids (MUFAs) feature a single carbon-carbon double bond (Figure 7), which may occur at different locations along the carbon chain [84]. PUFAs possess more than one double bond (Figure 7) [83].

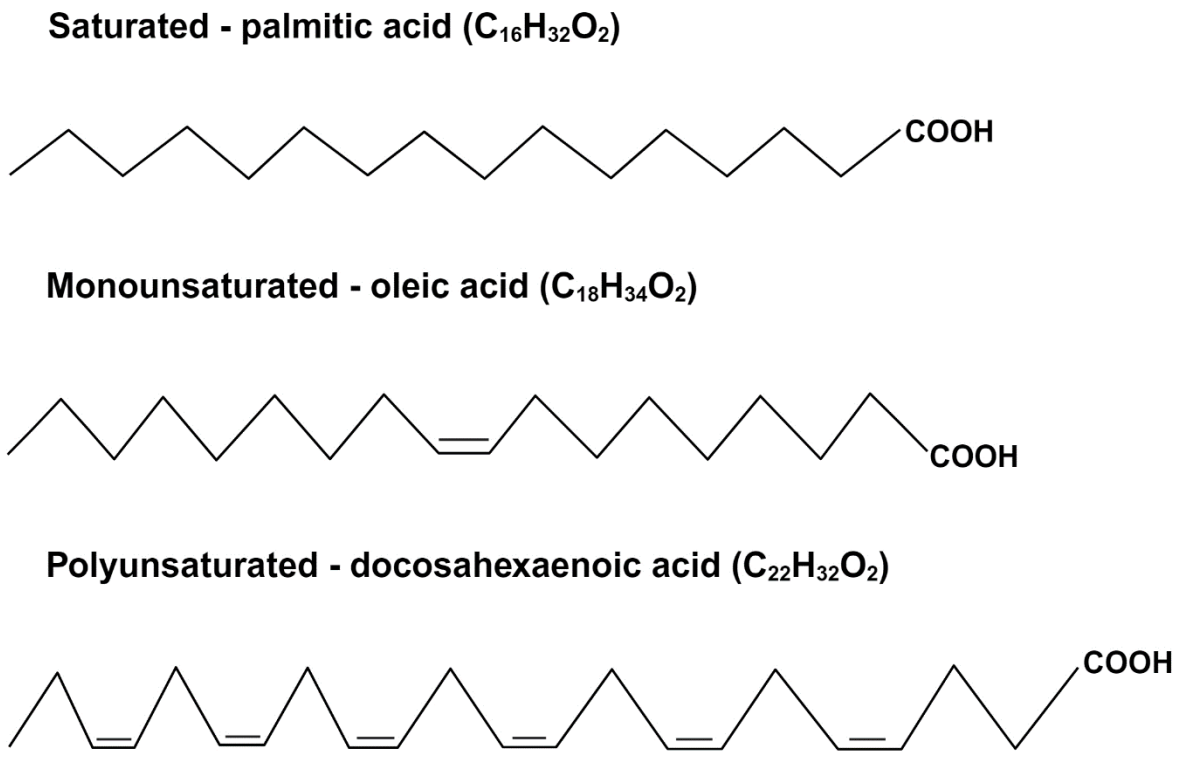

Figure 7. The key structural differences between saturated, monounsaturated, and polyunsaturated fatty acids lie in their chemical bonds*. Saturated fatty acids lack double bonds. In contrast, monounsaturated fatty acids possess one carbon-carbon double bond, whereas polyunsaturated fatty acids feature one or multiple double bonds. Illustration made by the author using BioRender.com.*

In PUFAs, the initial double bond may emerge either between the third and fourth carbon atoms from the omega carbon, referred to as omega-3 fatty acids, or between the sixth and seventh carbon atoms, designated as omega-6 fatty acids. A methylene (-CH2-) group separates the double bonds within PUFAs [83, 84]. These essential nutrients, generated solely by plants and phytoplankton, are vital for the health and functioning of higher organisms, including both mammals and fish. Omega-3 and omega-6 fatty acids cannot be converted interchangeably and are both deemed essential for biological processes [84].

1.3.1 Metabolism of fatty acids

When digested, fatty acids are absorbed within the small intestine [85]. Fatty acids are then transformed through re-esterification into triacylglycerols by the intestinal mucosa cells [86]. They travel through the lymphatic vessels to join the bloodstream in lipoprotein structures called chylomicrons [87]. Fatty acids in the bloodstream either bind to albumin or are conveyed within lipoproteins, facilitating their transport to various tissues and organs throughout the body [86].

PUFAs undergo additional metabolic processes involving the incorporation of carbon atoms through the action of elongases and the insertion of new double bounds thanks to the action of desaturases [88]. Mammals possess desaturases specialized in removing hydrogen atoms typically from carbon atoms positioned between an already existing double bond and the carboxyl group [89], and are unable to synthesize double bonds in fatty acids past the ninth carbon position (so ω-3 and ω-6 double bonds) [90].

In mammals, PUFAs, such as docosahexaenoic acid (DHA), are abundantly present in the testis and epididymis, playing crucial roles in sperm development and function [91-96]. While germ cells are richer in PUFAs content, Sertoli cells are more active in PUFAs metabolism [97]. The latter exhibit higher expression levels of Δ5- and Δ6-desaturases compared to germ cells [98], indicating their greater capacity for PUFAs synthesis. Notably, they seem to prefer omega-3 fatty acids for the synthesis of 22- and 24-carbon PUFAs over omega-6 [99]. This may provide insight into why sperm has a high DHA content [99]. Epididymal sperm maturation is essential for acquiring sperm functionality and involves remodeling the lipid composition of the sperm membrane. In mammals, fatty acid saturation increases from the caput to the cauda epididymis, while the proportion of PUFAs remains consistent throughout the epididymis [92, 93]. In rodents, desaturases and elongases are expressed in the epididymis [91, 98] In mice, epididymal spermatozoa contain higher levels of PUFAs compared to testicular sperm [92]. Additionally, during sperm maturation, optimal DHA levels are crucial, as a deficiency can impair acrosome formation [92, 100].

Cells primarily uptake fatty acids through passive diffusion and protein transporters embedded in the plasma membrane [101-103]. Fatty acid-binding proteins are responsible for the transport of fatty acids inside the cells [104]. Following uptake, free fatty acids undergo activation into acyl-CoA before being directed to either mitochondria or peroxisomes for beta-oxidation [104].

Beta-oxidation is a process that yields energy ultimately in the form of ATP. The process begins with the addition of coenzyme CoA to a fatty acid, resulting in Acyl-CoA [82]. Acyl-CoA binds with carnitine to enter the mitochondrial matrix as acyl-L-carnitines [82]. When inside they undergo reconversion into acyl-CoA [82]. In the matrix, acyl-CoA undergoes cycles of two-carbon unit cleavages, producing one molecule of acetyl-CoA, NADH and $FADH_2$ with each cycle [82]. Acetyl-CoA enters the citric acid cycle, where it undergoes oxidation to CO_2, producing ATP after oxidative phosphorylation [82].

The beta-oxidation of fatty acids is understood to be active in the testis. Studies conducted on rat models have shown the presence of high amounts of mitochondrial fatty acid beta-oxidation enzymes in both Leydig cells and Sertoli cells [105]. In the latter, lipid beta-oxidation is identified as a predominant pathway for ATP production [106].

In animal studies, fatty acids derived from the breakdown of phospholipids [107] can undergo oxidation within epididymal spermatozoa [108]. The analysis of the human sperm tail proteome revealed that 24% of the proteins involved in metabolism and energy production consist of enzymes engaged in lipid metabolism, including those governing mitochondrial beta-oxidation and the carnitine shuttle system [109]. This indicates a significant involvement of lipid metabolism pathways in sperm function. Furthermore, in spermatozoa across various species, the oxidation of fatty acids has been demonstrated to supply the energy necessary for motility [110-113]. A recent paper suggests that the inhibition of the phosphorylation of NF-κB inhibitor alpha (IκBα) in human spermatozoa by Bay117082, resulted in enhanced sperm motility indicating that fatty acids are utilized for energy [114], in addition to carbohydrates [115, 116].

1.3.2 Sperm fatty acids

Numerous studies have investigated the fatty acid composition of spermatozoa [19, 117-120]. The ratio between PUFAs, saturated fatty acids and cholesterol is correlated with sperm membrane fluidity [69] which is believed to play an important role in sperm motility [121].

The fatty acid profile of a sperm cell consists of approximately 50% SFAs, 15% MUFAs, and 35% PUFAs, with around 22% being omega-3 fatty acids and 13% omega-6 fatty acids [18, 119].

The most representative SFAs are palmitic acid (30-40%) and stearic acid (14-26%) [18, 119]. Zerbinati and colleagues found that sperm palmitic acids were linked to a lower risk of falling outside the normozoospermia range, while stearic acids were associated with reduced sperm motility [122]. However, it is important to note that the relationship between the SFAs class in spermatozoa and semen characteristics varies across different studies [118, 123-125].

The most representative PUFAs is DHA constituting approximately 20% of the sperm lipid composition [18, 123]. Studies have shown that sperm PUFAs, are positively correlated with sperm parameters [18, 19, 117, 118, 124] while MUFAs exhibit a negative correlation [18, 123].

1.4 L-carnitine

Carnitine, derived from amino acids, is integral to the metabolism of fatty acids [20]. While also biosynthesized in the liver and kidneys using lysine and methionine, the body largely relies on dietary sources for its carnitine requirements [126]. These sources include red meat, dairy products, and vegetables (with very low carnitine content) [126]. Carnitine exists as one of two stereoisomers: L-carnitine (Figure 8), biologically active and D-carnitine which is not biologically active in humans and can be toxic [127, 128]. In mammals, the carnitine reservoir is comprised of both free L-carnitine and L-carnitine bound to fatty acids [126]. L-carnitine uptake has been observed in multiple tissues, including the heart [129], skeletal muscle [130], liver [129] , kidney [129], epididymis [131-133], and brain [129].

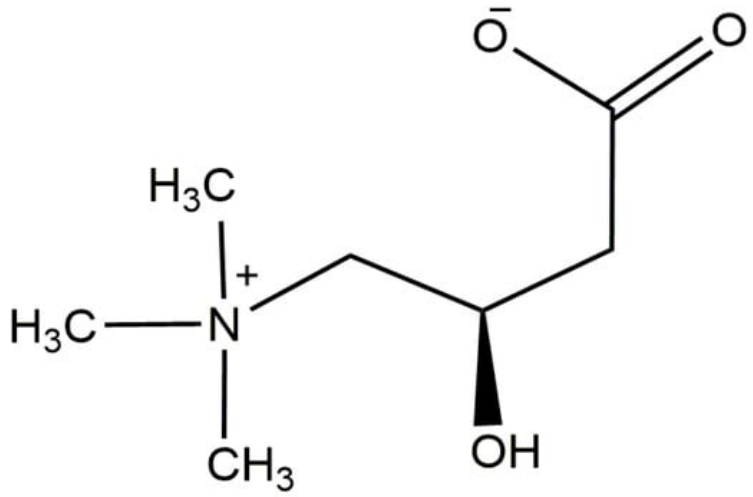

Figure 8. Molecular configuration of L-carnitine. *Figure retrieved from Durazzo et al., 2020 [134] under the CC BY 4.0 license.*

1.4.1 Metabolism of L-carnitine

Enzymes responsible for carnitine biosynthesis are widespread, with one exception: γ-butyrobetaine hydroxylase, the final enzyme in the pathway, which is notably absent in skeletal muscle and heart tissue [135]. This enzyme exhibits its highest activity levels in liver, kidney, and testicular tissues [135, 136].

Upon synthesis or ingestion, investigations into carnitine absorption and its constituents in animals, humans, and in vitro studies suggest a process where carnitine is transported from the intestinal lumen into enterocytes [126]. The portion of carnitine that bypasses the absorption in the small intestine undergoes substantial degradation by bacteria in the large intestine [126].

Carnitine is primarily removed from the body through renal excretion of both free L-carnitine and carnitine bound to fatty acids [126]. However, in typical circumstances, only a small percentage of carnitine is excreted. Under physiological circulating concentrations (25–50 μM), carnitine undergoes efficient reabsorption facilitated by the organic cation/carnitine transporter 2 (OCTN2) embedded in the apical membrane of renal epithelial cells [20, 126, 137]. Interestingly, as dietary carnitine intake decreases, the efficiency of its reabsorption increases. This delicate regulatory mechanism ensures that circulating carnitine levels remain within a normal range, enabling the elimination of excess carnitine [20, 126].

1.4.2 Distribution of L-carnitine in the male genital tract

Within the male reproductive tract of mammals, free L-carnitine accumulates at high concentrations from the systemic circulation into the epididymal lumen facilitated by OCTN2 and the carnitine transporter 2 (CT2) [138, 139]. L-carnitine has also been found in the testes, prostate, and seminal vesicles, although its concentrations are lower than in the epididymis [140]. Consequently, elevated levels of L-carnitine have been also observed in both spermatozoa and seminal plasma [64, 133]. This reservoir of L-carnitine is absorbed by spermatozoa through a combination of passive and active transport [133, 141, 142].

1.4.3 Functions of L-carnitine in the male reproductive system

The main function of L-carnitine is the transport of long-chain fatty acids to the mitochondria for beta-oxidation [20]. The carnitine shuttle system is schematically represented in Figure 9. Inside the cell, carnitine engages in the formation of a high-energy ester bond with long-chain fatty acids under the catalysis of carnitine palmitoyl transferase 1 (CPT-1), and with short-chain fatty acids under the action of carnitine acetyl-carnitine transferase, both situated in the inner layer of the outer mitochondrial membrane and found in mammal spermatozoa [143, 144]. This process culminates in the generation of acyl-carnitines [144]. The enzyme carnitine palmitoyl transferase 2 (CPT-2), situated in the inner mitochondrial membrane, catalyzes the removal of carnitine from acyl-carnitines, thereby regenerating acyl-CoAs [104, 144]. Carnitine can then return to the cytoplasm for another cycle thanks to the activity of an enzyme called carnitine-acylcarnitine translocase (CACT), while acyl-CoAs, under aerobic conditions and in the presence of low ATP levels, are eligible for entry into β-oxidation [144]. CACT has been identified in human testis and epididymis [145]. Free L-carnitine is also able to transport acetyl-CoA (one of the products of the beta-oxidation) out of the mitochondria helping to regulate excessive acetyl-CoA production which could alter the cellular metabolism [133, 146]. Acetyl-CoA can also enter Krebs (citric acid) cycle.

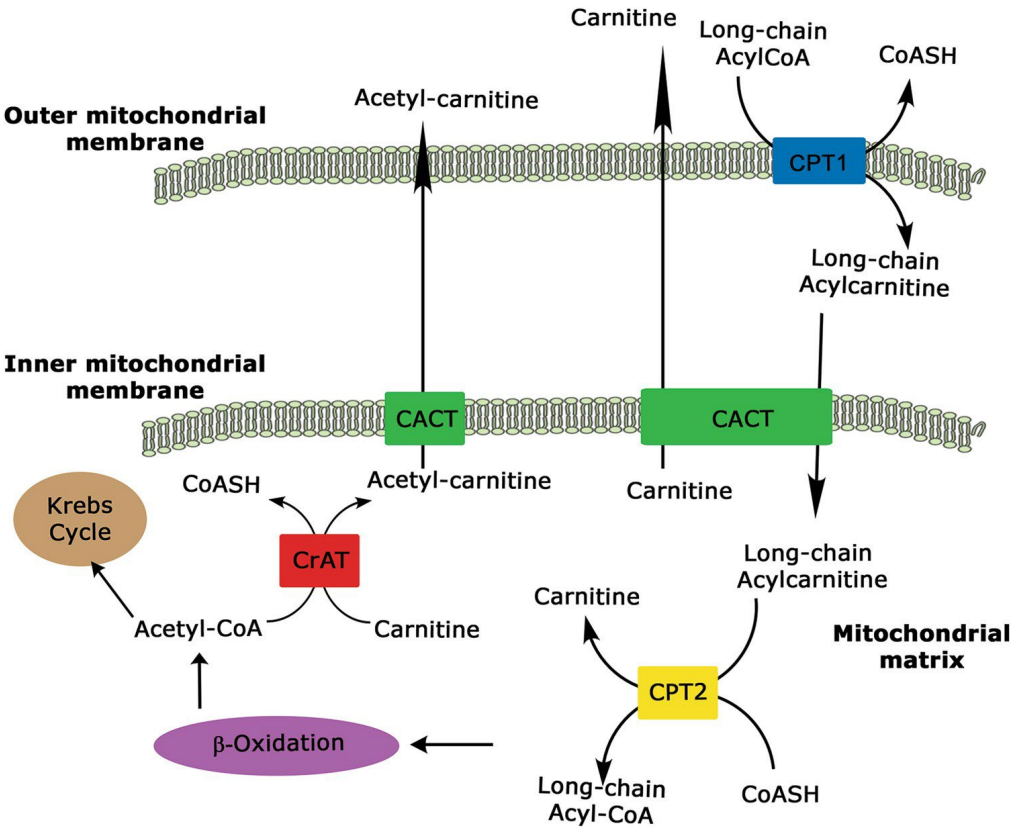

Figure 9. Carnitine shuttle system. *CPT1 converts long-chain acyl-CoA to acylcarnitine. Acylcarnitine is then translocated across the inner mitochondrial membrane by CACT. Within the mitochondrial matrix, CPT2 converts acylcarnitine back to long-chain acyl-CoA, which enters the β-oxidation pathway. CrAT facilitates the exchange of acetyl groups between CoA and carnitine. CPT1, carnitine palmitoyltransferase 1; CACT, carnitine-acylcarnitine translocase; CPT2, carnitine palmitoyltransferase 2; CrAT, carnitine acetyltransferase; CoASH, coenzyme A. Figure retrieved from Melone et al., 2018 [147] under the CC BY 4.0 license.*

L-carnitine also facilitates the transport of fatty acids between peroxisomes and mitochondria. Very long-chain fatty acids cannot directly enter mitochondria; instead, they undergo initial processing through peroxisomal beta-oxidation [148, 149]. Final peroxisomal β-oxidation products are transported to mitochondria, via carnitine, for complete oxidation and ATP production [150].

Additional physiological roles of carnitine involve modulating the acyl-CoA/CoA ratio [151, 152] and storing energy in the form of acetyl-carnitine [151, 153]. and mitigating the toxic effects of poorly metabolized acyl groups by binding them to form carnitine esters [146].

L-carnitine has been shown to have antioxidant properties [16, 154, 155] even though the precise mechanism has not yet been confirmed [154]. The antioxidant action of L-carnitine

involves several significant mechanisms. L-carnitine is a free radical scavenger and it is able to chelate transition metals such as iron (Fe) and copper (Cu) which are involved in ROS formation [154]. L-carnitine can also inhibit enzymes involved in the production of free radicals such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [154] and can also inhibit lipid peroxidation [156]. L-carnitine additionally can be regarded as a mitochondria-specific antioxidant, responsible for preserving mitochondrial integrity and modulating the production and signaling of ROS [154].

1.5 Impact of oxidative stress on sperm function and DNA integrity

Oxidative stress occurs when there is an imbalance between the production of ROS and the body's antioxidant defense systems [16]. Numerous cellular processes such as protein phosphorylation, activation of transcription factors, apoptosis, immunity, and differentiation rely on the precise regulation of ROS production and maintenance of their levels within cells at a controlled low level [16, 157]. However, an uncontrolled increase in ROS production can instigate detrimental effects on cellular components causing cellular dysfunction and compromising overall cellular integrity [16, 158].

1.5.1 Generation of reactive oxygen species by spermatozoa and semen

ROS production in spermatozoa can occur through two distinct avenues: via the NADPH oxidase system in the sperm plasma membrane, and/or through the nicotinamide adenine dinucleotide-dependent oxidoreductase reaction within the mitochondria. Since spermatozoa are rich in mitochondria, the latter mechanism might predominantly serve as the primary source of ROS generation [16].

O_2^- is the main ROS generated in human spermatozoa, which undergoes dismutation to generate H_2O_2 [159]. In the presence of metals like iron and copper, H_2O_2 and O_2^- engage in the Haber-Weiss reaction, producing $^{\bullet}OH$ radicals. These $^{\bullet}OH$ radicals can initiate a lipid peroxidation cascade, leading to impaired sperm functionality by altering membrane fluidity [159-161].

Sources of ROS can be both endogenous (leukocytes, immature spermatozoa, varicocele) and exogenous (radiation, toxins, smoking and alcohol consumption) [159].

PUFAs, highly present in sperm plasma membranes, are sensitive to lipid peroxidation which is one of the sources of ROS [159, 162]. Lipid peroxidation has the potential to compromise membrane fluidity and integrity, thereby influencing sperm structure and function (13, 14).

Spermatozoa have limited amounts of ROS-scavenging enzymes due to their small cytoplasmic volume. Thus, the main antioxidant defence system of the spermatozoa is found in the seminal plasma (19, 20). Seminal plasma is rich in enzymatic antioxidants such as catalase, superoxide dismutase, glutathione transferase, and glutathione peroxidase [163-165]. Complementing these enzymatic defences are non-enzymatic antioxidants such as vitamins E and C [166-168]. Furthermore, seminal plasma exhibits a robust buffering capacity, essential for balancing the acidic environment of the female reproductive tract [169, 170].

1.5.2 Sperm DNA damage

Normal spermatozoa exhibit chromatin structured with linear nucleotide arrangement and the absence of DNA strand breaks, modifications, or base loss [171, 172]. Sperm DNA fragmentation refers to the condition where the DNA within sperm cells is damaged or broken into fragments.

Chromatin damage includes defects such as DNA strand breaks, DNA base modifications, DNA cross-linkage, and protamine deficiency [172]. Specifically, sperm DNA fragmentation refers to breaks occurring within DNA strands, categorized as single-strand (SS-DBs) or double-strand breaks (DS-DBs). Sperm DNA fragmentation can arise during spermatogenesis, spermiogenesis, epididymal transit, or after ejaculation [172].

During spermiogenesis, the transition from histones to protamines takes place forming a condensed chromatin structure stabilizing the DNA and safeguarding the paternal genome [172, 173]. Any disruption in protamination can lead to faulty chromatin compaction [172]. Inadequate repair of DNA breaks during the transition from histones to protamines can lead to lasting DNA damage and induce cell death [172], which additionally makes sperm more susceptible to oxidative stress-induced DNA damage [172, 174].

Excessive generation of ROS during transit of spermatozoa through the seminiferous tubules and epididymis can also lead to sperm DNA damage [175]. While levels of oxidative stress below apoptosis-inducing thresholds can impair sperm functions, sperm with damaged

chromatin may still be able to fertilize the oocyte [172, 176]. If sperm DNA damage exceeds the oocyte's repair capacity, it can have a negative impact on embryo development and the well-being of the progeny [172, 177]. Findings from multiple meta-analyses published in recent years suggest potential adverse effects on embryo development, implantation, and pregnancy outcomes in both natural and assisted reproduction settings [178-181].

Varicocele, lifestyle choices, radiotherapy, chemotherapy, exogenous toxins, ageing and infections are causative factors of sperm DNA fragmentation [175].

1.6 Semen analysis and preservation

According to WHO, infertility is “a disease of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse” [182]. In one-third of infertile couples, the primary issue is with the male partner [183]. Male infertility and reduced semen quality can stem from a variety of factors, ranging from obstructive azoospermia to isolated abnormalities in seminal fluid or sexual and ejaculatory dysfunction. Furthermore, systemic illnesses, endocrine irregularities, iatrogenic complications, congenital anomalies, acquired damage to the testes, varicocele, immunological factors, infections of the male accessory glands and lifestyle factors can all contribute to the issue [3]. Although a significant portion of male infertility cases are still considered idiopathic [184]. For the assessment of male infertility, the analysis of ejaculated semen plays an important role, since sperm count is reflective of the functionality of the testes and their associated ducts, while the volume of seminal fluid offers insights into the activity status of the accessory sex glands. Semen analysis is often used by in vitro fertilization (IVF) clinicians as the first step when treating a couple, as its results provide valuable insights into the man's fertility potential and guide subsequent treatment decisions [3].

1.6.1 History of semen analysis

Concepts regarding the role of semen in human reproduction have ancient roots. Texts from ancient Greeks, Romans, Egyptians, explored theories on semen's role in reproduction, discussing the influence of seeds on offspring characteristics and hinting at infertility causes [185-189].

Many centuries later, thanks to the enhancement of the magnification capability of the microscope up to 300 times, Antony van Leeuwenhoek together with Johan Ham observed for the first time a human spermatozoon [189]. Leeuwenhoek detailed their findings in a letter to the Royal Society of London in November 1677, shedding light on seminal liquefaction, sperm motility, and morphological characteristics [189, 190]. Although rudimentary and lacking clinical applications, this marked the inception of semen analysis [189].

In 1824, Prévost and Dumas provided key evidence of the sperm's role in fertilization [189]. As understanding of reproduction grew in the 19th century, male infertility became recognized, leading to the need for semen analysis [189]. However, no lab tests existed then. Male reproductive issues were assessed mainly during the coitus, with little focus on semen parameters [189].

In 1866, Mantegazza was the first researcher who observed a correlation between semen characteristics and male fertility, primarily focusing on semen volume as an indicator [189, 191]. Additionally, he was the first to explore the impact of temperature on sperm motility. James Marion Sims built upon Mantegazza's work by introducing the analysis of progressive motile sperm in the cervical mucus post-coitus, through a laboratory test known as the postcoital test [189].

Later on, in 1891, Alois Lode made the first attempt to count spermatozoa using a Thoma-Zeiss chamber with semen diluted in potassium hydroxide [189]. He assessed sperm from both animals and humans, discovering a human sperm concentration ranging from 0 to 135 million/mL, with an average of 226 million sperm per ejaculate, which aligns with the results typically observed in contemporary routine semen analysis [189].

During this period, physicians agreed on the need to investigate the male partner in infertility diagnoses. However, such investigations typically occurred only if the female partner was sterile [189].

In 1915, Zeleny and Faust introduced a new technique for sperm morphology analysis that measured sperm head lengths under high magnification [189, 192]. 10 years later, Williams found a correlation between abnormal bull sperm morphology and head size using biometrical analysis of stained semen smears [189].

Although utilized in the 1920s and early 1930s, biometrical analysis of spermatozoa was gradually overshadowed by a focus on abnormalities in sperm head, midpiece, and tail. This was due to its time-consuming nature and criticisms for not measuring head width, possibly failing to identify links to sterility [189]. However, modern morphometric analysis is now integral to sperm morphology assessment in computerized systems [189].

During the early 1900s, Cary pioneered semen analysis standardization, “a historic milestone” [189, 193]. Hotchkiss expanded upon his work, establishing a clear benchmark for laboratory semen analysis [189, 194]. However, lingering problems remained, particularly regarding reference values for examination [189].

Despite standardizations in the 1930s, semen analysis remains controversial in predicting male infertility [189], although it remains the premier method for semen analysis [189].

1.6.2 Semen analysis in current days

Today, semen analysis adheres to WHO recommendations, which establish standardized procedures and reference values worldwide [5, 7]. The first edition of the WHO manual was released in 1980, followed by five subsequent editions spanning four decades. Most recently, the 6th edition was released in 2021 which provides updated information and guidelines, including reference values for semen characteristics (Table 1) and detailed methods for sperm analysis [5, 195]. Standard semen analysis comprises assessment of semen volume, sperm concentration, total sperm count, sperm motility, sperm morphology and sperm vitality [5].

Sperm motility and sperm concentration play an important role in the likelihood of conception and fertilization process. In a prospective study on couples planning their first-time pregnancy, Bonde and colleagues found that the likelihood of conception rises until sperm concentration reaches 40 million/mL [196]. In another prospective study, Zinaman and colleagues, observed a decline in pregnancy rates in healthy couples when sperm concentration was less than 30 million/mL [197]. The ability of spermatozoa to exhibit a progressive movement is essential for traversing through the cervical mucus, for navigating the uterine cavity, and ultimately, for reaching the fertilization site in the fallopian tube. Previous studies have in fact shown that sperm motility was positively associated with spontaneous pregnancy and fertilization and pregnancy rates in IVF [198-201].

Through sperm morphological analysis based on strict criteria, associations between the proportion of "normal" forms and various fertility outcomes, such as time to pregnancy and pregnancy rates both in vivo and in vitro, have been elucidated [202-206]. However, the complexity of the analysis and a lack of standardization across centres have introduced complexities in interpreting this parameter [207].

Sperm characteristics are also affected by ejaculatory abstinence which is the interval between the current semen sample and the previous ejaculation [5]. Sperm build up in the epididymis until capacity is reached, then pass into the urethra and are expelled during urination [5]. Since the epididymis is not completely emptied with a single ejaculation [5], residual sperm from prior ejaculations can influence the age and quality of sperm in the sample [5, 208], sperm vitality and DNA structure stay consistent despite changes in abstinence periods [209] except when epididymal function is compromised [5, 210]. Research shows that it typically takes 2-3 days of daily ejaculations to exhaust epididymal sperm reserves [5, 211, 212]. Consequently, the recommendation for men to abstain for 2-7 days before providing a sample might introduce variability [5].

Additional variables such as DNA fragmentation and oxidative stress tests can also be measured.

While semen analysis provides important information, relying solely on its results is inadequate for diagnosing male infertility [8].

Table 1. The lower reference values, 5th percentile with the 95% confidence interval (CI), of semen parameters of the fertile men.

Semen parameters	5th percentile	95% CI
Semen volume (mL)	1.4	(1.3-1.5)
Sperm concentration (10^6/mL)	16	(15-18)
Total sperm number (10^6/ejaculate)	39	(35-40)
Progressive motility (%)	30	(29-31)
Normal forms (%)	4	(3.9-4.0)
Vitality (%)	54	(50-56)

To derive the WHO 2020 distribution values, Campbell and colleagues integrated data from 2010 with that from 2010-2020, culminating in an analysis of up to 3,589 subjects (Analysis 2: WHO 2020). Table retrieved from Campbell et al., 2021 [195] under the CC BY 4.0 license and modified by the author.

1.6.3 Methods for assessing DNA fragmentation and oxidative stress

Several DNA fragmentation tests have been developed, each with its own set of advantages and limitations [213]. The Sperm Chromatin Structure Assay (SCSA), based on flow cytometry, is widely regarded as the premier method for evaluating DNA fragmentation in spermatozoa, having undergone extensive validation across human and livestock species [214, 215]. It assesses sperm DNA vulnerability to acid-induced denaturation, employing the dye acridine orange (AO), which binds to intact double-stranded DNA, causing the cells to emit green fluorescence; however, damaged single-stranded DNA binding results in red fluorescence [216]. It can detect both SS-DBs and DS-DBs and can analyse a substantial number of spermatozoa, ranging from 5000 to 10,000, within seconds [172, 217]. Nevertheless, its reliance on costly equipment and specialized expertise may pose considerable obstacles to its widespread adoption [213].

The Tunel assay (Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling) is a technique for detecting DNA fragmentation associated with apoptotic processes [218]. This assay operates on the principle that the enzyme terminal deoxynucleotidyl transferase (TdT) labels the 3'-OH ends of fragmented DNA with modified nucleotides [219]. The TUNEL assay can detect both SS-DBs and DS-DBs by flow cytometric or fluorescence microscopic analysis [218].

The Comet assay (single-cell gel electrophoresis) is a technique for detecting DNA strand breaks at the level of individual cells. This assay is based on the principle that fragmented DNA

migrates out of the cell nucleus under an electric field, forming a pattern resembling a comet, with a distinct head (intact DNA) and tail (damaged or fragmented DNA) [220]. Comet assay is a sensitive technique for identifying minimal DNA damage [221]. It is capable of differentiating between viable, apoptotic and necrotic cells. However, only DS-DBs are detectable with this technique and it is not possible to observe small deletions [221].

Sperm Chromatin Dispersion (SCD) test evaluates the structure of sperm DNA by examining the central core and peripheral halo formed due to the release of DNA loops [222]. When spermatozoa containing intact DNA are treated with lysing solutions in an agarose matrix, deproteinized nuclei or nucleoids display halos of dispersed DNA [223]. These halos are observable under both bright field and fluorescence microscopy [223]. Spermatozoa with DNA fragmentation typically lack this halo formation [223]. SCD is considered a straightforward and cost-effective method and finds extensive utilization in fertility clinics [224]. SCD has been validated in several species [223, 225-227], however, its validation for implementation in bull sperm remains pending.

Recently, a novel method called STRIDE (Sensitive Recognition of Individual DNA Ends) has been developed for quantifying DNA breaks in fixed cells using fluorescence microscopy [228]. Two protocols are available for this technique: one for SS-DBs, called sSTRIDE, and another for DS-DBs, termed dSTRIDE. dSTRIDE offers the ability to assess double-strand DNA breaks at the single-cell level [228].

It is noteworthy that the diagnostic thresholds, also referred to as cut-off values, for these methodologies are inherently specific to the individual assay and the particular methodology employed during its execution [172].

Elevated levels of ROS in seminal plasma have been associated with reduced sperm count [229] and motility [230, 231], as well as an increase in DNA fragmentation [232]. The occurrence of sperm DNA damage, whether induced by oxidative stress or other etiologies, can lead to adverse reproductive outcomes such as pregnancy failure or reproductive pathologies [159, 160].

Numerous alternative research methods are at the disposal to directly analyse ROS levels in the ejaculate such as luminol, oxidation-reduction potential (through Mioxsys [233]), and total antioxidant capacity analyses [5, 234]. However, as per WHO 2021, “from a clinical diagnostic

perspective, this group of assays should only be used and interpreted with caution until more conclusive proof of their diagnostic relevance exists" [5].

1.6.4 Sperm cryopreservation

Sperm cryopreservation is commonly used for male fertility preservation, as well as for assisted reproductive technologies such as IVF and intrauterine insemination [9]. The procedure is also used for donor sperm storage [11], prior to cancer treatments [10], in case of autoimmune diseases [235] and upon testicular and epididymal sperm extraction for assisted reproductive technologies (ART) [236]. However, the process of cryopreservation can have deleterious effects on sperm quality, which can ultimately impact fertility outcomes. The damage to sperm occurs during both the freezing and thawing processes, which induces cold shock, osmotic stress, intracellular ice crystal formation, and oxidative stress [15]. These mechanisms can lead to changes in sperm morphology, motility, viability and DNA structure [12, 14].

To counteract these detrimental effects, cryoprotectants (CPAs) are incorporated into the solution before freezing. CPAs, categorized into two types based on their ability to permeate cell membranes, function by reducing the freezing point of intracellular and extracellular water, shielding cells from mechanical injury caused by ice crystal formation during freezing and thawing [9]. Additionally, the composition of a CPA helps maintain optimal osmotic pressure, safeguarding spermatozoa from damage due to abrupt changes in osmotic pressure during freezing and thawing [9, 237]. Glycerol was the first used CPA [238], followed by dimethyl sulfoxide [239], which penetrates cells faster and requires a lower concentration to avoid the detrimental effect of freezing/thawing processes [9]. Toxicity concerns prompted the development of alternative CPAs like sugars (monosaccharides and disaccharides) which due to their size cannot permeate cellular membranes [9, 237].

Additives with antioxidant properties can enhance the action of CPAs, limiting the detrimental effects of ROS on spermatozoa [9]. Several studies have shown that antioxidants protect sperm during cryopreservation, with improved sperm motility, viability, and acrosomal integrity [240-244]. For example, Trolox, a water-soluble vitamin E analogue, has been shown to increase sperm motility [240] when added to cryoprotectant solutions, while vitamin C has been shown to protect sperm against ROS during cryopreservation [241]. Cryopreservation media supplemented with L-carnitine has demonstrated efficacy in mitigating damage inflicted by the freezing process on spermatozoa, resulting in enhanced semen quality after thawing [24, 25]

along with a reduction in ROS levels within the spermatozoa [26]. Additionally, melatonin, a powerful antioxidant, has been shown to improve sperm motility, viability, and reduce ROS following cryopreservation [242]. Similarly, cysteine, a sulfur-containing amino acid, has been reported to protect sperm against cryoinjury by scavenging ROS and stabilizing the sperm membrane [243]. Overall, using antioxidants in cryopreservation can potentially improve sperm quality and fertility outcomes, making it a promising area of research.

The speed of freezing and thawing significantly impacts sperm survival post-cryopreservation, minimizing ice crystal formation and osmotic pressure exposure. Two main techniques, rapid freezing and vitrification, are utilized by IVF clinics. Rapid freezing involves direct contact with CPAs and exposure of the carrier with the sample to nitrogen vapour and subsequent immersion in liquid nitrogen at a temperature of -196 °C [245]. In vitrification, sperm cells are immersed in high concentrations of CPAs and exposed to rapid cooling and warming rates, effectively preventing ice formation [9]. While both techniques use similar CPAs, vitrification requires higher concentrations of them. Li and colleagues found that vitrification had a superior sperm recovery rate than rapid freezing, though both methods exhibited similar DNA fragmentation levels [246]. While vitrification is simpler and more cost-effective than rapid freezing, its limited freezable volume makes rapid freezing the primary choice for sperm cryopreservation worldwide.

1.7 Animal sperm as a model system

Animal models have played a crucial role in unravelling the core principles of male reproductive biology and shaping innovative strategies for preserving fertility [27]. Initial refinement of techniques such as sperm cryopreservation, intracytoplasmic sperm injection (ICSI) and IVF occurred through animal studies [27]. Rodents, with their rapid reproduction and comprehensively studied reproductive systems, have been especially influential, while larger animals such as rabbits, bovines, and primates provide a more analogous physiological context to humans [27-29]. Besides methodological reasons, animal models allow researchers to explore hypotheses and conduct experiments that may not be feasible or ethical in human subjects [247]. Animal semen is more homogenous and therefore easier to use for experiments. Moreover, studying sperm in animal models can be more cost-effective than human studies, enabling researchers to conduct a broader range of experiments and investigations. This cost-effectiveness facilitates the exploration of various hypotheses and experimental designs to

advance our understanding of sperm biology. Animal models also serve as an educational tool for training diverse reproductive techniques [27]. However, it's important to recognize their constraints, as they incompletely mimic human physiology, demanding cautious interpretation of findings from such models [27]. Table 2 outlines examples and summarizes the advantages and limitations of key animal models frequently employed in fertility studies.

Table 2. Animal models in reproductive biology and male fertility: a comprehensive overview.

Animal Models	Classifications	Contributions	Advantages	Limitations
Caenorhabditis elegans	Nematodes	Exploring primary reproductive mechanisims	Affordable, low maintenance requirement, rapid reproductive cycles [248]	Distantly related to humans on the evolutionary scale
Fruit fly	Insects	Exploring primary reproductive mechanisims	Affordable, low maintenance requirement, easy to manipulate [249]	Distantly related to humans on the evolutionary scale
Zebrafish	Fish	Exploring primary reproductive mechanisims	Affordable, low maintenance requirement, high degree of similarity with human genome, similar anatomy of germ cell organs to that in humans [250]	Distantly related to humans on the evolutionary scale
Mouse	Mammals, rodent	Sperm cryopreservation [251], development of ART technologies [252], genetic studies in fertility [253], reproductive toxicology [254], germ cells transplantation [255]	Affordable, low maintenance requirement, rapid reproductive cycles, wide range of diseases model available	The germline in both humans and mice varies in several aspects
Rat	Mammals, rodent	Sperm cryopreservation [256], reproductive toxicology [254], germ cells transplantation [255]	Affordable, low maintenance requirement	ART models not developed as in mice
Rabbit	Mammals	Sperm cryopreservation [257], development of ART technologies [258], reproductive toxicology [259]	Similar reproductive system to humans	Higher maintenance cost than rodents
Bovine	Mammals	Sperm cryopreservation [32], development of ART technologies [260], sexed semen [261]	Good breeding, fertility and progeny data records	High maintenance cost
Non-human primate	Mammals	Sperm cryopreservation[262], training in reproductive procedures [263]	Highly similar to human on the evolutionary scale	Expensive and complex care, ethical and regulatory constraints

Table retrieved from Aponte et al., 2024 [27] under the CC BY 4.0 license and modified by the author. ART, assisted reproductive technologies.

1.7.1 Influence of animal models in human reproductive technologies: historical perspectives

Development in animal reproductive technologies has greatly influenced human infertility procedures [27, 264]. In the 1770s first artificial inseminations were performed in animals and humans thanks to the scientific contributions of Lazzaro Spallanzani and John Hunter who is recognized as a pioneer in human artificial insemination [27, 265, 266]. In the 1800s, artificial insemination was reported to be used in other mammals [27, 265]. With the development of the first artificial vaginas, Milovanov started cattle breeding which paved the way for the first large-scale bovine artificial insemination organization established in Denmark in 1936 [27]. The storage and transportation of animal semen was made possible by the development of semen extenders in the mid-1900, laying the ground for Christopher Polge's work which demonstrated glycerol's protective role in sperm cryopreservation, revolutionizing the field [27, 32]. This milestone culminated with the birth of the first calf conceived through insemination with frozen-thawed semen in 1950s [27, 32]. In the 1970s, You and colleagues introduced computerized systems for the evaluation and processing of bull semen which was then extended to various species, including humans [27, 267]. In the 1980s, the development of technologies for sorting X- and Y-carrying spermatozoa marked a significant breakthrough [27, 261]. Commercial availability of sexed semen for cattle followed in the 1990s [27, 268]. In vitro fertilization and embryo transfer techniques developed first on animal models, have opened the door for the establishment of IVF techniques in humans which culminated in the world's first human IVF pregnancy and the birth of Louise Brown occurred in Oldham, UK in 1978 [27, 269].

1.7.2 Bull spermatozoa

Bull spermatozoa is a valuable research model within the realms of reproductive biology, genetics, and biomedicine. By studying bull spermatozoa, researchers can gain insights into the complex interplay between genetic factors, sperm physiology, and reproductive outcomes.

Morphologically, bull spermatozoa are similar to the rest of the mammalian spermatozoa. The average head of a bovine spermatozoon is 9.1 μm, the midpiece 13,6 μm and the tail 47 μm [270]. The total length of a bovine spermatozoon is therefore circa 70 μm and similar in size to sperm of humans and monkeys, 60 and 70 μm respectively [271]. The heads of bovine

spermatozoa are less spherical than humans but still more spherical than porcine and mouse spermatozoa [270].

One of the key advantages of utilizing bovine spermatozoa in research is their capacity to produce high amounts of spermatozoa suitable for extensive experimentation and analysis. Semen quality is largely determined by the selection of the bull [272]. Bull selection programs worldwide aim to ensure that bulls are physically sound, free from reproductive or genetic defects, and highly fertile. Bull fertility plays a crucial role in cattle reproduction [273]. Its importance surpasses that of cows due to the ability of a single bull to inseminate hundreds of thousands of cows through artificial insemination [273]. Even though bull semen parameters can vary among breeds, exceedingly, on average bulls produce an ejaculate volume of around 8 mL with 67 % progressive motility and a sperm concentration of over 1000 mill/mL [30]. A normal human semen ejaculate typically exhibits lower volume, sperm concentration, and motility compared to the bovine ejaculate.

Experimentation analyses with bull spermatozoa contributed and can further contribute to advancements in assisted reproductive technologies, contraceptive development, and infertility treatments, in both humans and animals [27, 274]. Innovations in sperm cryopreservation and in vitro fertilization techniques have revolutionized the field of reproductive science, opening new avenues for experimental investigation and clinical application [27, 275].

In addition, bull spermatozoa are valuable tools for environmental and toxicological studies [276, 277]. By exposing bull spermatozoa to various environmental stressors and toxicants, researchers can assess the impact of environmental contaminants on male fertility and reproductive health. Studies in this area provide critical insights into the effects of toxins, pollutants, pesticides, and industrial chemicals on sperm function, viability, DNA integrity and ROS informing strategies for environmental conservation and human health protection [31-33, 276, 277].

2. Aims of the study

L-carnitine plays a crucial role in transporting long-chain fatty acids for β-oxidation and contributes to antioxidant defense. It is also found in high concentrations in the epididymis and ejaculate, where it positively correlates with sperm concentration and motility in seminal plasma. Given these factors, the overall aim of this project is to evaluate the impact of seminal endogenous L-carnitine on sperm parameters in humans. Furthermore, we investigated alternative methods for DNA fragmentation using bull spermatozoa as a study model. The project is characterized by the following objectives:

- To examine the relation between the amount of distribution of free L-carnitine in the seminal plasma and levels of sperm fatty acids. Furthermore, we wanted to explore if BMI is associated with the levels of seminal L-carnitine.

- To examine the impact of endogenous levels of free seminal L-carnitine on semen parameters after freezing-thawing. We also investigated the effect of freezing medium supplemented with L-carnitine on post-thaw semen parameters, related to endogenous seminal L-carnitine levels.

- To investigate the use of Sperm Chromatin Dispersion (SCD) and Sensitive Recognition of Individual DNA Ends (dSTRIDE) in the assessment of DNA damage in bovine sperm, as compared to Sperm Chromatin Structure Assay (SCSA).

3. Summary of the papers

Paper I:

Levels of L-carnitine in human seminal plasma are associated with sperm fatty acid composition

Authors: Mario Iliceto, Mette Haug Stensen, Jorunn M. Andersen, Trine B. Haugen, Oliwia Witczak

The functionality of spermatozoa is significantly influenced by their fatty acid composition, with L-carnitine playing a key role in fatty acid metabolism. Elevated levels of L-carnitine in seminal plasma are positively associated with semen quality. In contrast, a high BMI is negatively associated with semen quality and those sperm fatty acids important for sperm function. Here we investigated the associations between free seminal L-carnitine levels and sperm fatty acid composition as well as BMI. Semen samples were collected and analysed from 128 men with unknown fertility status and with BMI ranging from 19 kg m^{-2} to 63 kg m^{-2}. Gas chromatography was used to analyse sperm fatty acid composition and free seminal L-carnitine levels were measured with high-performance liquid chromatography. The results from multiple linear regression analysis revealed a positive correlation between free seminal L-carnitine levels and sperm palmitic acid, docosahexaenoic acid, and total n-3 polyunsaturated fatty acids. Free seminal L-carnitine levels showed a negative correlation with lignoceric acid and total n-6 polyunsaturated fatty acids, after adjusting for covariates. No significant relationship was found between free seminal L-carnitine levels and BMI. These findings indicate that while free seminal L-carnitine levels are linked to semen quality, the lack of correlation with BMI suggests that reduced semen quality in obesity is independent of L-carnitine levels.

Paper II:

Association of endogenous seminal L-carnitine levels with post-thaw semen parameters in humans

Authors: Mario Iliceto, Mette Haug Stensen, Jorunn M. Andersen, Trine B. Haugen, Oliwia Witczak

Cryopreservation of semen serves as an important method for preserving male fertility. Seminal L-carnitine is associated with good semen quality. Here we wanted to explore the relationship between naturally occurring levels of seminal L-carnitine and post-thaw semen parameters. Additionally, we investigated how adding L-carnitine to the freezing medium could impact sperm characteristics, in relation to the endogenous levels of L-carnitine in semen. The research encompassed the analysis of 125 fresh semen samples, alongside post-thaw samples that underwent standard cryopreservation and cryopreservation with L-carnitine supplementation. Participants were divided into two groups based on the median levels of endogenous seminal L-carnitine: low (≤38.8 µg/mL) and high (>38.8 µg/mL). Results indicated that after standard cryopreservation, samples with high levels of L-carnitine exhibited better sperm motility and lower oxidative stress compared to samples with low levels. On the other hand, post-thaw samples with low L-carnitine levels showed increased sperm neck midpiece defects compared to fresh samples. The study also found that cryopreservation with L-carnitine supplementation had the most significant positive impact on rapid progressive sperm motility in samples with high endogenous L-carnitine levels. Overall, the findings suggest that L-carnitine, whether naturally occurring in seminal plasma or added as a supplement in the freezing medium, enhances sperm characteristics by improving motility and reducing oxidative stress in post-thaw samples.

Paper III:

Assessment of alternative methods to Sperm Chromatin Structure Assay for evaluating DNA damage induced by oxidative stress in bull sperm

Authors: Ingvild Aas, Mario Iliceto, Else-Berit Stenseth, Anne Hege Alm-Kristiansen, Oliwia Witczak, and Erwan Delbarre

In mammals, sperm DNA damage is linked to decreased fertilization ability and has been shown to adversely affect fetal development in offspring. The Sperm Chromatin Structure Assay (SCSA) is widely regarded as the benchmark for assessing sperm DNA integrity, including detecting single-strand and double-strand breaks. Nonetheless, the high cost of this method often prevents many fertility clinics and breeding organizations from utilizing it. In this study, the focus was on exploring alternative methods to the Sperm Chromatin Structure Assay (SCSA) for assessing DNA damage in bovine sperm. Through incubating five semen samples with concentrations of H_2O_2 ranging from 0 to 100 mM, the study aimed to evaluate DNA damage using SCSA, SCD, and a newly developed method called Sensitive Recognition of Individual DNA Ends (dSTRIDE). Results showed that both SCSA and SCD effectively detected a significant increase in DNA damage in spermatozoa exposed to high H_2O_2 concentrations ($\geq$ 10 mM), compared to controls. However, dSTRIDE did not detect such increases in DNA fragmentation under these conditions. The study suggests that SCD could be a viable alternative to SCSA, while dSTRIDE requires further investigation for its efficacy in assessing DNA damage in bovine semen.

4. Discussion

4.1 Methods

4.1.1 Human and animal study population

In the first paper, men over the age of 18 years were recruited for participation from the general population (70%), fertility (19%), and obesity clinics (11%). This recruitment strategy was designed to include a diverse participant pool, yet it might introduce potential variability in the characteristics of the participants. Participants from the general population joined the study voluntarily and they might not be representative of the broader population in terms of health behaviors and socio-cultural background [278]. Men were recruited from obesity clinics to obtain a broader BMI distribution. Those participants were undergoing or preparing for surgical intervention due to severe obesity and their participation may be influenced by their current health status and medical necessity, which could impact their overall lifestyle and health metrics differently than those of the general population. Similarly, men from fertility clinics may have different health and fertility-related issues compared to those from the general population, potentially influencing semen quality and other fertility parameters. To achieve a wider age distribution in the group of men with a BMI ≤ 27 kg/m², we included normal-weight men from fertility clinics, only those where the couple was diagnosed with only female factor infertility. This cutoff was chosen because many of our potential participants had a BMI slightly above the normal weight range (BMI > 25 kg/m²), necessitating a practical adjustment to include a broader pool of relatively lean individuals who were on the lighter side of the overweight category but not obese.

In the second paper, men aged 18 years and older were recruited from a fertility clinic in Oslo. The recruitment primarily included men who were not part of couples undergoing IVF treatment, accounting for 89% of the participants. This approach was likely intended to ensure a broad representation of male fertility status without the specific influence of ongoing fertility treatments, even though we did not record the history of infertility of the participants, thus potentially confounding our results. Incomplete samples, as per patient information, were excluded from the study, along with samples with a sperm concentration of less than 5 million per milliliter and/or progressive motility of less than 10%. These exclusions might introduce limitations to the generalizability of our findings even though they might help to maintain the integrity of the data. In fact, excluding samples with very low sperm parameters could help

clarify the relationship between seminal L-carnitine levels and post-thaw semen quality. This approach might reduce confounding effects such as skewed L-carnitine to sperm ratios due to low sperm count, which could otherwise mask the relationship between L-carnitine and sperm quality.

In the third paper, bull semen samples were sourced from the Norwegian bull breeding company Geno AS. In this study, we analysed semen samples from one Charolais bull and four Norwegian Red bulls. Although specific comparisons between Norwegian Red and Charolais bulls are not well-documented in the literature, it is known that semen quality parameters can vary between breeds [279]. These breed-specific differences could affect the generalizability of the study's findings, even though no differences in DNA fragmentation have been observed between breeds [279].

4.1.2 Study design

The first paper has a cross-sectional design, commonly used for analyzing relationships between variables [280]. A limitation of this study design is the potential for non-responders to have poorer lifestyle habits, which could lead to an underestimation of the prevalence of adverse lifestyle factors among the men studied [281]. While questionnaires are inherently subjective, in our study anthropometric data were collected by trained personnel, to minimize potential bias. Because cross-sectional studies, like ours, capture data at a single moment in time, they present challenges in determining causal relationships [280]. However, they provide foundational insights that are valuable for designing a more comprehensive future study [280].

The second and third papers are in vitro studies. This type of study design offers controlled conditions that enable researchers to minimize external influences and examine the effects of the supplement, providing insights into causality. However, biases can still arise, potentially affecting the validity and reliability of the results [282]. For instance, unintentional biases in sample selection or measurement procedures could impact study outcomes. In our case, we were aware of which samples underwent the intervention and which did not, potentially biasing our interpretation of the results. However, employing a blinded approach was unfeasible due to logistical constraints, since the analysis was conducted by a single researcher, making implementation of such a method impractical.

4.1.3 Ethical considerations

The research detailed in the first and second papers received approval from the Regional Committee for Medical and Health Research Ethics (REK; reference number: 2018/423), South East, Norway, with all participants providing written informed consent. However, for the third paper, as the bulls were part of regular semen production, ethical committee approval was deemed unnecessary.

4.1.4 BMI measurement

In our study, we used BMI for the measurement of adiposity. BMI is calculated by dividing the weight in kilograms (kg) by the height in square meters (m^2). In the first paper, we divided men into tertiles based on their BMI distribution (18.8–25.8 kg/m²; 25.9–31.2 kg/m²; and 31.3–62.7 kg/m²) to ensure an equal number of participants in each group.

BMI, due to its simplicity, is widely used to assess overweight status [283]. However, BMI does not account for body fat distribution [283], and this can lead to incorrect categorization of individuals who possess more muscular mass and/or larger skeletal structures [283].

In the second paper, BMI was calculated based on self-reported data from the participants of the study. It is important to note that self-reported data can introduce bias in the BMI measurements. It has been observed that BMI measurements from self-reported data are typically underestimated [284].

4.1.5 Semen analysis

The research outlined in the first paper commenced in 2008, and semen analysis was performed in accordance with the 1999 edition of the WHO manual [285]. The assessment of sperm motility was recorded via video files using guidelines outlined in the 2010 WHO manual [7].

The research described in the second paper started in 2018 where concentration, motility, and vitality were performed in adherence to the guidelines outlined in the 2010 WHO manual [7]. However, the assessment included the evaluation of rapid progressive motility, a part of the categorization system specified in the WHO 1999 manual [285].

The study published in the third paper started in 2023 and semen concentration of the bulls was performed using a Neubauer improved chamber which is considered the standard for sperm cell counting [5].

Even if manual semen analysis is considered the gold standard, it is susceptible to subjectivity. To address this limitation, laboratory personnel were trained in semen analysis techniques, with regular external quality controls coordinated by the European Society of Human Reproduction and Embryology.

In paper II all the participants who decided to produce the semen sample at home delivered it to the laboratory within 1 hour after collection in accordance with the WHO guidelines. However, in the first paper, we extended this time limit to 2 hours after collection. Even though this delay in analyzing samples brought from home could introduce bias into our study, the majority of the samples (77%) were delivered to the laboratory within 1 hour after collection.

In our study sperm morphology was assessed in accordance with the Tygerberg strict criteria [286]. However, in the second paper sperm smear was stained using SpermBlue and not using the Papanicolau staining method which we used in the first paper, and it is recommended in the WHO guidelines. However, the Papanicolaou staining method is time-consuming, and due to logistical constraints, it was deemed unfeasible and impractical to implement in our research setting. We opted for a more rapid staining method such as SpermBlue, also because, according to the literature, the head length and width measurements of SpermBlue-stained sperm tend to be most consistently similar to those of fresh native spermatozoa [287].

4.1.6 Fatty acid analysis

Sperm fatty acids were analysed by gas chromatography at Vitas AS, a private laboratory in Oslo. Gas chromatography is highly suitable for the detection of fatty acids in tissues and cells including spermatozoa [18, 117]. However, there may still be limitations in detecting fatty acids at very low concentrations [288]. For the derivatization process, methanol extraction was followed by HCl derivatization, which is able of detecting cholesterol esters [289] which are also present in spermatozoa [290]. Since the fatty acids were isolated from the cell pellet after centrifugation, fatty acid content from other cell type could have influenced our study. However, only 7% of semen samples contained round cells.

4.1.7 Analysis of redox potential in semen samples

We utilized the MiOXSYS system to measure the total static oxidative-reductive potential (sORP), also known as redox potential, in semen samples. As per the manufacturer's guidelines, the sORP (mV) value generated automatically by the MiOXSYS reader needs to be divided by the sperm concentration of the samples to calculate the normalized sORP per 10^6 sperm/mL. In our study, we expressed sORP in mV. The reason for not expressing sORP in mV/10^6 sperm/mL is grounded in recent publications [291, 292]. According to Castleton and colleagues, normalizing with the sample concentration reveals that correlations between normalized ORP and other sperm parameters are influenced by sperm concentration—a crucial variable in assessing sperm quality [293]. The study highlights that associations between redox potential and sperm parameters, such as motility, are driven solely by adjustments for sperm concentration [293].

4.1.8 Seminal L-carnitine analysis

Free L-carnitine in the seminal plasma samples was analysed using liquid chromatography/tandem mass spectrometry. The analysis was conducted by a commercial laboratory based in Oslo. This method is becoming increasingly prevalent in laboratory procedures for analyzing low molecular weight molecules [294]. It has been employed in various studies for detecting L-carnitine in seminal and blood plasma samples, as evidenced by prior research [294-298].

In the first paper, sample collection was performed several years before the analysis of seminal L-carnitine. However, the samples were kept in aliquots in an ultra-low -80ºC freezer which are used for long-term storage of biological materials and have been shown to maintain the quality level required for biochemical analysis [299]. Freeze-thaw treatment is the critical step. All samples were treated equally by trained personnel. The samples were never subjected to thawing before the analysis of free L-carnitine was performed, and the thawing was performed on ice.

4.1.9 Semen cryopreservation

In the second paper, following the initial semen analysis, each sample was divided into two equal aliquots for cryopreservation: one aliquot was cryopreserved using standard sperm

freezing medium, while the other was preserved in medium supplemented with 0.5 mg/mL L-carnitine. This concentration was chosen after testing the impact of a range of L-carnitine concentrations (0, 0.1, 0.5, 1, 10, and 25 mg/mL) on sperm motility in 10 semen samples. The results indicated that the addition of 0.5 mg/mL L-carnitine to the freezing medium yielded the most favorable effect on post-thaw sperm motility. Notably, this selected concentration aligns with findings from a prior investigation [24]. In our study, sperm motility was the sole parameter used to assess the effects of various L-carnitine concentrations, following the approach taken by others for different supplements [300, 301]. In contrast, other research groups have employed a broader range of parameters, including sperm membrane integrity, vitality, and DNA damage, in addition to motility [302-304].

4.1.10 DNA fragmentation analysis

In the first and third papers, we employed SCSA to evaluate DNA fragmentation in human spermatozoa, as it is widely regarded as the gold standard for sperm DNA analysis [305]. In the second paper, we used the SCD test instead of SCSA. Although SCD is not considered the gold standard, it produced results comparable to those of the SCSA test [306, 307].

In the third paper, where a novel method for DNA fragmentation (dSTRIDE) was tested using bull sperm, both SCD and SCSA were utilized. The experimental design of this study included the addition of H_2O_2 to induce oxidative stress and DNA fragmentation in bull sperm. H_2O_2 is a well-known oxidative agent that generates ROS, leading to DNA strand breaks [308]. This approach was chosen because it mimics the oxidative conditions that spermatozoa might encounter in vivo, thereby providing a relevant model for assessing the reliability of DNA fragmentation tests under oxidative stress conditions.

Martinez-Pastor and colleagues conducted a similar study and found that SCD was less reliable than SCSA for measuring DNA fragmentation in bull sperm treated with oxidative agents (Fe^{2+}) [309]. However, the kit they used (SPERM-HALOMAX) was different from ours (Goldcyto DNA). Unlike SPERM-HALOMAX, Goldcyto DNA kit includes an acidic denaturation step, making it more similar to the SCSA method. The SPERM-HALOMAX method relies solely on protein depletion treatment. The authors noted that the lysis solution used in the SPERM-HALOMAX test alters chromatin differently compared to the acid-detergent used in SCSA, disintegrating the protein scaffold, and potentially explaining the differences between the two

techniques. In summary, the different measurement conditions for DNA fragmentation by these two kits, complicate direct result comparison.

4.1.11 Statistics

Statistical analyses were performed using multiple linear regression (as discussed in Paper I) and partial correlation (as detailed in Paper II) to account for potential confounders.

In Paper I, since some samples collected at home were delivered in more than one hour after ejaculation, we included time to analysis as a covariate because it can influence sperm motility [285]. In contrast, in Paper II we did not adjust partial correlation for time to analysis because all participants who collected samples at home did so within one hour.

In Paper I, men from the fertility clinic were included to achieve a broader age distribution within the group of men with a BMI ≤27 kg/m². A subanalysis was conducted to investigate whether there were differences in free seminal L-carnitine levels and semen parameter values between participants recruited from the general population and those from the fertility clinic. The analysis revealed no significant differences in levels of free seminal L-carnitine between the two groups.

In Paper II, participants were categorized into two groups based on the 50th percentile (median) of seminal L-carnitine levels: low L-carnitine (≤38.8 µg/mL) and high L-carnitine (>38.8 µg/mL). To our knowledge, no publications have yet identified a specific cut-off for seminal L-carnitine content. In non-parametric data, dividing groups based on the median, as previously done in studies by Staníková and colleagues and Terraneo and colleagues [310, 311], provides valuable insights. This approach splits the data set into two halves, allowing for a clear comparison between the lower and upper halves of a specific molecule. However, these studies were not focused on L-carnitine.

In Paper III, we refrained from normality testing of the data due to the inherent limitations of such tests when dealing with small datasets. Instead, we opted for non-parametric methods, which bypass the assumption of normality and are often more suitable in such scenarios. Our approach involved presenting the data as median and quartiles, utilizing the Wilcoxon Signed-Rank test for pairwise comparisons, and employing adjustments for multiple comparisons, such as the Bonferroni method, to maintain the validity of statistical inferences [312].

To evaluate the ability of SCSA, SCD, and dSTRIDE to measure varying degrees of DNA fragmentation in bovine sperm, we exposed spermatozoa from five different bulls to increasing concentrations of H_2O_2, ranging from 1mM to 100mM. The analysis was conducted in triplicates for SCD and duplicates for SCSA and dSTRIDE. The samples processed using the dSTRIDE protocol were analysed with fluorescence microscopy, and only a limited number of cells were counted giving a potentially skewed representation of the overall sample.

4.2 General Discussion

4.2.1 Seminal L-carnitine and sperm fatty acids

PUFAs are vital components of spermatozoa membranes and play a critical role in sperm function [69, 119]. To our knowledge, the relationship between free seminal L-carnitine levels and sperm fatty acid content in humans has not been previously explored. Previous studies have shown that sperm PUFAs and in particular DHA are associated with semen quality [18, 19, 117, 118]. In mammals, high DHA concentrations are found in the cauda epididymis, where mature spermatozoa are stored, compared to other segments [313-316]. L-carnitine, known to have a central role in cellular aerobic metabolism by transporting long-chain fatty acids across the mitochondrial membranes for beta-oxidation, is also abundant in epididymal fluid, and its levels have been found to positively correlate with sperm parameters such as progressive sperm motility and sperm count [22, 23]. Moreover, dietary supplementation with L-carnitine improves semen quality in infertile men [317] and increases total n-3 PUFAs, enhancing semen quality in roosters [318]. In our study, we found that free seminal L-carnitine levels positively correlated with total PUFAs, total n-3 PUFAs, palmitic acid, and DHA levels, and negatively correlated with total n-6 PUFAs and lignoceric acid levels in spermatozoa.

Our results emphasize the critical role of sperm fatty acid composition in humans and suggest a specific role for seminal L-carnitine in sperm maturation within the epididymis. The exact mechanism is unclear, but two possible explanations are proposed. Firstly, L-carnitine may enhance sperm motility by facilitating the oxidation of fatty acids, providing necessary energy. Secondly, fatty acids released from maturing sperm might signal the upregulation of carnitine transport genes (OCTN2 and OCTN3) in the epididymal epithelium, thereby regulating L-carnitine transport from the bloodstream into the epididymal lumen.

It should be noted that this study measured only free, not total or acetylated L-carnitine levels in seminal plasma and did not assess L-carnitine in spermatozoa. Additionally, seminal fatty acid content was not analysed, which could have provided further insights into the relationship between seminal L-carnitine and sperm fatty acids, as well as the fatty acid composition of the epididymal epithelium, especially considering the exchange of L-carnitine between seminal plasma and spermatozoa [133].

4.2.2 Impact of L-carnitine on semen parameters in both fresh and post-thaw samples

In Papers I and II, we observed a positive association between seminal L-carnitine levels and sperm parameters such as concentration and progressive motility, and a negative association with semen volume, consistent with prior research [22, 23, 319]. However, we did not find significant associations with sperm vitality or morphology, contrary to findings by Zofpgen and colleagues [23]. Comparing semen quality studies is challenging due to differences in semen analysis methods [320] and intra- and inter-technician individual variability [321].

The relationship between endogenous seminal L-carnitine levels and post-thaw semen quality has not been extensively studied. In Paper II, our findings indicate that semen samples with higher endogenous seminal L-carnitine levels had a greater amount of motile sperm post-thaw compared to samples with lower levels. This observation, while not proving causality, suggests that high levels of seminal L-carnitine may mitigate the detrimental effects of cryopreservation.

In this study, we also observed an increase in oxidative stress in post-thaw samples compared to fresh samples, regardless of the L-carnitine levels, as evidenced by elevated seminal sORP levels. However, this increase was less pronounced in samples with high endogenous seminal L-carnitine levels, aligning with the known antioxidative properties of L-carnitine.

Cryopreservation is linked to sperm mitochondrial dysfunction, lipid peroxidation, and decreased sperm motility and morphology [12, 14, 322]. Our results showed that sperm neck and midpiece defects were more prevalent in post-thaw samples with low seminal L-carnitine levels. In contrast, no significant difference in neck and midpiece defects was observed in fresh samples, regardless of L-carnitine levels, suggesting a protective effect of L-carnitine during

cryopreservation. Given the established association between oxidative stress and reduced sperm motility due to lipid peroxidation, our findings—lower sORP levels and higher motility in post-thaw samples with high L-carnitine—support the role of endogenous seminal L-carnitine in reducing oxidative stress in cryopreserved samples. It should be noted that we did not assess mitochondrial function or lipid peroxidation in our study. However, previous research has demonstrated a positive correlation between sperm mitochondrial dysfunction and the prevalence of sperm midpiece defects [14], while increased oxidative stress has been linked to reduced sperm motility due to lipid peroxidation [231].

In our investigation, consistent with prior research [37, 38], we found that supplementing the freezing medium with L-carnitine yielded positive effects on post-thaw semen quality. Moreover, our study revealed that the enhancement in rapid progressive sperm motility following cryopreservation with L-carnitine was particularly pronounced in samples exhibiting high levels of endogenous seminal L-carnitine, in contrast to those with lower levels.

It should be noted that the effect of L-carnitine supplementation on post-thaw sperm motility grades was not uniformly beneficial across all samples when compared to standard cryopreservation without L-carnitine supplementation. Notably, samples with low endogenous L-carnitine levels exhibited a greater variation in sperm motility grades than those with high L-carnitine levels. This variability suggests that the post-thaw outcomes of L-carnitine supplementation are more unpredictable in samples with low endogenous L-carnitine levels. Cryopreservation has been shown to reduce seminal superoxide dismutase (SOD) activity, with significant inter-individual variation among donor samples [323]. Reduced SOD activity can lead to increased lipid peroxidation, compromising sperm membrane integrity and motility [323, 324]. Although we did not measure lipid peroxidation or seminal SOD activity, the observed variability in motility changes might be linked to differential SOD activity levels in individual semen samples. In samples with low endogenous L-carnitine, the baseline antioxidant capacity might be insufficient to counteract the oxidative damage induced by cryopreservation, leading to greater variability in motility outcomes. Conversely, high endogenous L-carnitine levels could provide a more robust antioxidative defense, resulting in more consistent post-thaw motility outcomes. This hypothesis aligns with our findings and underscores the importance of individual antioxidant capacity in determining the efficacy of cryopreservation protocols.

4.2.3 Seminal L-carnitine and BMI

High BMI may contribute to the reduced semen quality observed in obese men [325] and it has been shown to negatively correlate with those sperm fatty acids important for sperm function [19]. Given the essential role of L-carnitine in lipid metabolism and the fact that BMI might be associated with an altered fatty acid metabolism [326, 327], we explored the potential correlation between free seminal L-carnitine levels and BMI but did not identify any significant association. To our knowledge, no other studies have examined seminal L-carnitine levels specifically in overweight or obese individuals. Additionally, the literature on blood serum L-carnitine levels and BMI presents conflicting findings [328-330], making direct comparisons challenging, particularly since our study did not include blood sample measurements.

4.2.4 DNA fragmentation in bovine semen samples assessed by SCD, SCSA and dSTRIDE

This study aimed to compare the effectiveness of SCD and dSTRIDE with SCSA in assessing DNA fragmentation in bovine semen samples. The comparison focused on evaluating how each method responded to sperm exposure to oxidative damage induced by H_2O_2. A significant difference in DNA damage was observed when spermatozoa were exposed to high concentrations of H_2O_2 when assessed using both SCSA and SCD methods, but not with dSTRIDE.

SCSA results indicated a statistically significant increase in DNA fragmentation index (%DFI) at H_2O_2 concentrations above 1 mM. This method also showed a dose-response relationship, particularly between 1 mM and 10 mM concentrations, but the overall increase in % DFI was relatively modest (1.3-fold) at the lowest concentration compared to controls. SCD, on the other hand, exhibited a much higher sensitivity, detecting a 6-fold increase in DNA fragmentation at the same 1 mM concentration. The median % DNA fragmentation measured by SCD was significantly higher than that measured by SCSA, especially at higher H_2O_2 concentrations (10 mM and 100 mM). These findings align with a previous study that compared SCD and SCSA on human sperm [223].

To the best of the authors' knowledge, only one other study has compared SCSA and SCD methods in bull semen [309]. As mentioned earlier (4.1.10) the SCD method differs from the method used in our study, making the results difficult to compare. However, in humans, several

studies have shown a significant positive correlation between SCSA and SCD [223, 306, 307, 331]. The SCD methods employed in these studies, as well as in ours, are based on the SCD test developed by Fernandez and colleagues [222, 223].

dSTRIDE was investigated in the present study as an alternative method to SCSA for better insight into the amount of DS-DBs as dSTRIDE was presented as a method that selectively investigates DS-DBs [228]. In contrast to SCSA and SCD, the dSTRIDE method did not detect an increase in DNA fragmentation in sperm samples exposed to H_2O_2. This outcome can be explained by the nature of H_2O_2-induced DNA damage, which predominantly causes single-strand breaks rather than double-strand breaks. Given that dSTRIDE specifically targets DS-DBs, its lack of sensitivity to H_2O_2-induced damage is consistent with the understanding that H_2O_2 is more likely to produce SS-DBs than DS-DBs [332].

5. Conclusion

Our study demonstrated that free seminal L-carnitine levels correlated positively with levels of fatty acids in spermatozoa important for sperm function, including palmitic acid, DHA, and total n-3 PUFAs. Importantly, seminal L-carnitine levels were not associated with BMI, suggesting that the reduced semen quality observed in obesity is independent of L-carnitine levels. Our findings support the hypothesis that the epididymis plays a crucial role in sperm maturation and that beta-oxidation might be a vital source of energy for mature spermatozoa.

Secondly, we confirmed the beneficial effects of L-carnitine on semen quality in post-thaw samples. As an endogenous component of seminal plasma and a supplement in freezing media, L-carnitine has shown to positively impact sperm motility and reduce oxidative stress. Furthermore, higher endogenous levels of L-carnitine were associated with lower incidences of sperm neck midpiece defects following cryopreservation. This underscores the protective role of L-carnitine against the adverse effects of freezing.

Finally, our comparative study of methods for assessing DNA fragmentation revealed that both SCSA and SCD effectively detected significant increases in DNA damage in bull spermatozoa exposed to high concentrations of H_2O_2. In contrast, dSTRIDE did not detect increased DNA fragmentation under the same conditions. These findings suggest that while SCD can be considered a viable alternative to SCSA for assessing DNA damage, dSTRIDE requires further refinement and validation.

6. Future perspectives

Future research should employ causal study designs to determine whether free seminal L-carnitine influences the fatty acid composition of spermatozoa and to explore its role in energy production through fatty acid oxidation and oxidative phosphorylation in sperm cells. This could involve in vitro experiments using isolated spermatozoa to explore the metabolic pathways influenced by L-carnitine.

Future work should delve deeper into the mechanisms of how L-carnitine exerts its protective effects on spermatozoa, particularly during the cryopreservation process. One promising direction is to explore the molecular pathways influenced by L-carnitine that enhance sperm motility and reduce oxidative stress. This could involve examining the role of L-carnitine in mitochondrial function.

Given that findings in recent years suggest potential adverse effects of sperm DNA fragmentation on embryo development, implantation, and pregnancy outcomes in both natural and assisted reproduction settings, it is also important to explore and compare the different methods for sperm DNA fragmentation. Affordable and easy-to-use methods that fit well into routine clinical practice could be particularly valuable. Since the methods are often performed under different conditions, comparing the results can be difficult. dSTRIDE is a promising method for assessing DNA fragmentation and could benefit from further evaluation after double-strand brake induction, such as exposure to ionizing radiation, which could validate its utility in assessing genetic damage in spermatozoa.

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Papers I-III

Paper I

Levels of L-carnitine in human seminal plasma are associated with sperm fatty acid composition

Mario Iliceto, Mette Haug Stensen, Jorunn M. Andersen, Trine B. Haugen, Oliwia Witczak

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ORIGINAL ARTICLE

Male Fertility

Levels of L-carnitine in human seminal plasma are associated with sperm fatty acid composition

Mario Iliceto[1,2], Mette Haug Stensen[1], Jorunn M Andersen[2], Trine B Haugen[2], Oliwia Witczak[2]

The fatty acid composition of spermatozoa has been shown to be important for their function, and L-carnitine is crucial for fatty acid metabolism. Its levels in the seminal plasma positively correlate with semen quality, whereas high body mass index (BMI) is associated with both reduced semen quality and altered sperm fatty acid composition. Here, we examined the associations between free seminal L-carnitine levels and sperm fatty acid composition as well as BMI. Semen samples were collected and analyzed from 128 men with unknown fertility status and with BMI ranging from 19 kg m^{-2} to 63 kg m^{-2}. Sperm fatty acid composition was assessed by gas chromatography, while free seminal L-carnitine analysis was performed using high-performance liquid chromatography. Multiple linear regression analysis showed a positive correlation of free seminal L-carnitine levels with the amount of sperm palmitic acid (β = 0.21; *P* = 0.014), docosahexaenoic acid (DHA; β = 0.23; *P* = 0.007), and total n-3 polyunsaturated fatty acids (β = 0.23; *P* = 0.008) and a negative correlation of free seminal L-carnitine levels with lignoceric acid (β = –0.29; *P* = 0.001) and total n-6 polyunsaturated fatty acids (β = –0.24; *P* = 0.012) when adjusted for covariates. There was no relationship between free seminal L-carnitine levels and BMI. Since free seminal L-carnitine levels are associated with semen quality, the absence of a correlation with BMI suggests that reduced semen quality in obese men is independent of seminal L-carnitine.

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Keywords: body mass index; L-carnitine; male fertility; semen quality; sperm fatty acids; spermatozoa

INTRODUCTION

Among the many metabolic changes related to obesity, changes in semen quality including total sperm count,[1–3] concentration,[1–4] motility,[2,3] morphology, and vitality[3,4] have been observed. The composition of sperm fatty acids is also influenced by obesity.[5] In obese men, levels of sperm polyunsaturated fatty acids (PUFAs) including docosahexaenoic acid (DHA; C22:6 n-3) are reduced. Interestingly, the sperm fatty acid constituents are important for their function.[5–8] The levels of sperm PUFAs with 22 carbon atoms or more correlate positively with semen quality.[8] The sperm fatty acid composition has been shown to be modified during spermatogenesis and epididymal maturation;[9] however, its regulation in spermatozoa is not well understood.

L-carnitine, a ubiquitous quaternary ammonium cation derived from lysine and methionine, plays a vital role in fatty acid metabolism by transporting long-chain fatty acids to the mitochondria for beta-oxidation.[10] In mammalian cells, carnitine is present as both free L-carnitine and acylcarnitine esters produced by carnitine acyltransferases using various fatty acids as substrates. L-carnitine also prevents oxidative stress by regulating nitric oxide levels, cellular respiration,[11] and the activity of enzymes involved in the defense against oxidative damage.[12] Abnormalities in carnitine regulation are implicated in complications including diabetes mellitus and malnutrition.[13,14] Most of L-carnitine in humans is derived from dietary intake,[15] and carnitine supplementation has been shown to have beneficial effects on obesity and weight loss.[16] However, the association between body mass index (BMI) and serum L-carnitine levels in humans shows conflicting results.[17–19]

In the male reproductive tract of mammals, free L-carnitine is transported by the organic cation/carnitine transporter 2 (OCTN2) and the carnitine transporter 2 (CT2) from the systemic circulation into the epididymal lumen,[20,21] where it accumulates[22–24] and could play a role in sperm maturation and metabolism.[25] High levels of L-carnitine originating from the epididymis are found in both spermatozoa and seminal plasma.[26,27] The uptake of free L-carnitine into epididymal spermatozoa is suggested to be both passive[26] and active, mediated by OCTN1, OCTN2, and OCTN3.[28,29] In animal studies, fatty acids from the breakdown of phospholipids[30] can be oxidized in epididymal spermatozoa in a carnitine-dependent manner.[31] Jeulin *et al.*[32] reported parallel increases in the percentage of spermatozoa with progressive motility and free L-carnitine contents of distal corpus spermatozoa of the boar. Furthermore, in humans, levels of free seminal L-carnitine have been found to positively correlate with progressive sperm motility, sperm count,[23,33] and sperm morphology.[33] In addition, a study suggests that L-carnitine-mediated oxidation of fatty acids in the human spermatozoa is involved in motility.[34]

The aim of this study was to examine the relation between the amount of free L-carnitine distribution in the seminal plasma and levels of sperm fatty acids. Furthermore, we hypothesized that one of the mechanisms behind reduced semen quality observed in men with high BMI could result from altered seminal L-carnitine levels.

[1]Fertilitetssenteret, Oslo 0176, Norway; [2]Department of Life Sciences and Health, OsloMet – Oslo Metropolitan University, Oslo 0130, Norway.
Correspondence: Mr. M Iliceto (marioi@oslomet.no)
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PARTICIPANTS AND METHODS

Study population

Men over the age of 18 years were recruited between August 2008 and December 2013 in South-Eastern Norway as previously described.[3] Upon entry, weight (in kg) of the participants was recorded using a digital scale (Soehnle Professional, Backnang, Germany). Height (in cm) was measured by a wall-mounted stadiometer. In addition, information about medical treatment, dietary supplements, as well as history of cryptorchidism or previous cancers, which were exclusion criteria, was recorded. No prior knowledge of semen quality was required, and no further criteria were required for inclusion. Samples with spillage were excluded. For the present study, semen samples for L-carnitine analysis from 128 men were included, of which 90 participants were recruited from the general population by advertisement, 24 men were recruited from couples with female factor infertility from a fertility clinic (Fertility Department Soer, Telemark Hospital Trust, Skien, Norway) and 14 men from obesity clinics (The Morbid Obesity Centre, Vestfold Hospital Trust, Tønsberg, Norway, and Department of Morbid Obesity and Bariatric Surgery, Oslo University Hospital, Oslo, Norway). Men from the fertility clinic were included to achieve a wider age distribution in the group of men with BMI ≤27 kg m^{-2}, while men from the obesity clinic were included to obtain a broader BMI distribution. The study was approved by the Regional Committee for Medical and Health Research Ethics (REK), South East, Norway (REK number: 2008/3957 and 2010/2721), and all participants provided written informed consent.

Blood sampling and biochemical analysis

Fasting blood samples were collected before 10 a.m. Venous samples were centrifuged 30 min after collection at 1800*g* for 10 min, and serum and ethylenediaminetetraacetic acid (EDTA)-plasma were aliquoted and frozen at −80°C and stored until further analyses were performed. Levels of low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol, and triglycerides (TG) were measured in the serum, while glycated hemoglobin (HbA1c) was measured in the blood at Fürst Medical Laboratory, Oslo, Norway. Upon arrival, fasting glucose concentration was analyzed in 105 capillary samples using HemoCue 201+ Glucose Analyzer (HemoCue, Ängelholm, Sweden) at OsloMet (Oslo Metropolitan University, Oslo, Norway) and in 77 venous samples at Fürst Medical Laboratory. In fifty samples, glucose levels were analyzed using both methods, and since strong correlation between the two methods was found ($r = 0.939$), results from both were included in the analysis.

Semen analysis

Semen analysis was performed at OsloMet in the period of 2008–2013 according to the World Health Organization (WHO) guidelines[35] as previously described.[3] Semen samples were obtained by masturbation and collected on-site or at home in a preweighed sterile container. Participants were asked to abstain from ejaculating for 2–7 days before sample collection and to report the length of abstinence time. When collected at home, participants were asked to avoid cooling of the sample during transportation. For participants ($n = 24$) with long travel distance to the laboratory, the sample was delivered after more than 1 h. Out of 128 semen samples, 83 were collected on-site, 39 were collected at home, and the place of collection was not registered for 6 samples. Time to semen analysis was recorded. Out of 128 semen samples, 104 were analyzed within 1 h, 16 between 1 h and 2 h, and 8 before 4 h. Motility was not assessed in samples that were analyzed after more than 2 h. Semen samples were analyzed after completion of the liquefaction: on-site-collected semen samples were incubated for 30 min at 37°C, while samples collected at home were incubated for 10 min at 37°C. The ejaculated volume was estimated by sample weight. Sperm concentration was determined using a Neubauer improved hemocytometer (Hecht Assistant, Sondheim vor der Rhön, Germany) and morphology was determined according to the Tygerberg strict categorization[36] after Papanicolaou staining.[35] Laboratory personnel performing semen analysis at OsloMet participates in the external quality program for semen assessment organized by the European Society of Human Reproduction and Embryology. Sperm pellets for fatty acid analysis and seminal plasma for free L-carnitine analysis were prepared from the remaining ejaculate by centrifugation at 500*g* for 15 min. The samples were stored at −80°C.

Sperm chromatin structure assay

Sperm chromatin structure assay (SCSA) was performed in 2013 at Reproductive Medicine Center, Skåne University Hospital, Malmö, Sweden. The method is described previously.[3,37] Briefly, DNA fragmentation index (DFI) in the spermatozoa was analyzed using FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), equipped with an air-cooled argon ion laser. A total of 10 000 events were accumulated for each measurement at a flow rate of 200–300 cells s^{-1}. Flow cytometric data were analyzed by the SCSA Soft software (SCA Diagnostics, Brookings, SD, USA). All samples were run in the same batch by one technician with an intralaboratory coefficient of variation (CV) of 4.5%.

Fatty acid analysis

Fatty acid levels in the spermatozoa samples were analyzed in 2015, by gas chromatography at a commercial laboratory in Oslo (Vitas AS, Oslo, Norway) as previously described.[5] Briefly, frozen sperm pellets were thawed, methylated with 3 mol l^{-1} methanolic HCl, and neutralized with 3 mol l^{-1} KOH in water. Fatty acid methyl esters were extracted with hexane and analyzed on an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA). A SP-2380 column (Supelco, Bellefonte, PA, USA) was used for separation. Data were obtained for the most relevant and/or abundant individual fatty acids of spermatozoa[5–7] and are presented in **Supplementary Table 1**. Categories of fatty acids were also calculated including saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and PUFAs. PUFAs were additionally divided into n-3 and n-6 categories (**Supplementary Table 1**). Results for fatty acids are expressed as weight percentage (wt%) and accounted for 87.3 wt% of the total amount of fatty acids analyzed by gas chromatography.

Free L-carnitine analysis

Free L-carnitine in the seminal plasma samples were analyzed in 2018 for the purpose of this study using liquid chromatography/tandem mass spectrometry. Seminal plasma stored at −80°C was used and analysis of the samples was performed at a commercial laboratory in Oslo. A total of 10 µl seminal plasma was thawed at room temperature and diluted with labeled internal standard L-carnitine d9 (Sigma Aldrich, Taufkirchen, Germany) before undergoing butylation by methyl chloroformate and butanol. Samples were liquid-liquid extracted before analysis on an Agilent 1260 HPLC coupled to an Agilent 6460 QQQ mass spectrometer operated in Multiple Reaction Monitoring Mode (Agilent Technologies). Separation was performed on a Phenomenex Kinetex C18 100 mm × 4.6 mm × 2.7 µm analytical column (Phenomenex Inc., Torrance, CA, USA), and quantification was performed using a 7-point calibration curve covering a range of 5–200 µg ml^{-1}. Results for free seminal L-carnitine are expressed in µg ml^{-1}. Total free L-carnitine in

the seminal plasma (in μg per ejaculate) was calculated by multiplying free seminal L-carnitine concentration with semen volume.

Statistical analyses

All participant variables were summarized as median (range). BMI was calculated as weight in kg divided by height in meters squared. For the background characteristics, we categorized men into tertiles on the basis of the distribution of BMI (18.8–25.8 kg m^{-2}; 25.9–31.2 kg m^{-2}; and 31.3–62.7 kg m^{-2}) in order to maintain an equal number of participants in each group. Statistical differences in these characteristics by BMI tertiles were assessed by Kruskal–Wallis test. Total free L-carnitine in the ejaculate was entered in the analysis of background characteristics to account for interindividual differences in sex gland fluid contribution. To visualize differences in the sperm DHA levels and BMI across the total free L-carnitine distribution, total free L-carnitine levels were categorized into tertiles (<110.8 μg per ejaculate, 110.8–218.6 μg per ejaculate, and >218.6 μg per ejaculate), and the data were presented as Box–Whiskers plot. The Mann–Whitney U test was used to compare differences between the tertiles.

Variable distributions were evaluated by histograms. Skewed variables were log-, square-root-, squared-, or inverse-transformed. Multiple linear regression analysis was used to estimate associations between levels of free seminal L-carnitine and levels of sperm fatty acids and between levels of free seminal L-carnitine and semen characteristics. All variables were continuous, except for time to semen analysis that was dichotomized.

To estimate associations between levels of free seminal L-carnitine and levels of sperm fatty acids, covariates were included in the multivariate models if they were statistically significant at $P < 0.1$. Covariates included in the model were age (in year), sexual abstinence time (in day), and BMI (in kg m^{-2}), as these values varied among the participants. Associations between levels of free seminal L-carnitine and semen characteristics were also adjusted for time to semen analysis (≤1 h or >1 h), since not all samples were delivered and analyzed within 1 h after ejaculation. Motile sperm concentration (MSC; 10^6 ml^{-1}) was obtained by multiplying the sperm concentration and the percentage of motile spermatozoa divided by 100%. The MSC variable was entered in the regression analysis.

The Mann–Whitney U test was used for (i) comparison of free seminal L-carnitine levels in the samples delivered and analyzed within 1 h after ejaculation or later, (ii) comparison of free seminal L-carnitine levels and semen parameter values between participants recruited from the general population and from the fertility clinic, and (iii) comparison of age, free seminal L-carnitine levels, and semen parameter values between participants from the fertility clinic and a subgroup of men from the general population with comparable BMI ≤27 kg m^{-2}.

The level of statistical significance was set at 0.01 or lower. Statistical analyses were performed by using IBM SPSS Statistics 20 (IBM, Chicago, IL, USA).

RESULTS

Study population

The characteristics of the study population are described in **Table 1**. Only nine participants (7.0%) reported use of dietary supplements; however, none of the supplements included L-carnitine. When stratified by BMI tertiles, the factors closely related to obesity varied across the groups as expected, apart from total cholesterol. Neither free nor total free seminal L-carnitine levels showed difference across BMI tertiles. Among the men in the first (18.8–25.8 kg m^{-2}) and second (25.9–31.2 kg m^{-2}) BMI tertile, respectively, 18 (42.8%) and 6 (13.9%) were recruited from the fertility clinic from couples with female factor infertility. Fourteen (32.6%) men in the third BMI tertile (31.3–62.7 kg m^{-2}) were from the obesity clinic. The remaining men were from the general population.

Subanalysis was performed to investigate if free seminal L-carnitine levels were different in samples that were delivered and analyzed within 1 h after ejaculation or later (**Supplementary Table 2**). No such difference was observed.

Men from the fertility clinic were included in the study population to achieve a wider age distribution in the group of men with BMI ≤27 kg m^{-2}. Subanalysis was done to examine whether there were differences in free seminal L-carnitine levels and semen parameter values between participants recruited from the general population ($n = 104$) and those from the fertility clinic ($n = 24$; **Supplementary Table 3**). Levels of free seminal L-carnitine did not differ between the two groups. Significant differences in semen parameter values were only seen in the length of sexual abstinence time ($P = 0.004$) and in proportion of spermatozoa with normal forms ($P = 0.001$).

In addition, subanalysis was also performed to compare age, seminal L-carnitine levels, and semen parameter values between men from the fertility clinic and men from the general population with comparable BMI ≤27 kg m^{-2} ($n = 33$; **Supplementary Table 4**). As expected, there was a significant difference in age ($P < 0.001$), as men from the fertility clinic were older. There was no difference in seminal L-carnitine levels nor in semen parameter values between these two groups, except for seminal volume ($P < 0.001$) and sexual abstinence time ($P = 0.008$).

Table 1: Background characteristics of men in the study population by BMI tertiles

Variable	*BMI tertiles (kg m^{-2}), median (range)*			*P*
	Lowest tertile (n=42)	*Middle tertile (n=43)*	*Highest tertile (n=43)*	
BMI (kg m^{-2})	23.3 (18.8 – 25.8)	28.2 (25.9 – 31.2)	34.7 (31.3 – 62.7)	<0.001*
Age (year)	35 (24 – 51)	37 (22 – 59)	39 (22 – 61)	0.007*
TG (mmol l^{-1})	0.8 (0.4 – 5.2)[b]	1.1 (0.6 – 10.7)	1.6 (0.8 – 3.3)	<0.001*
Total cholesterol (mmol l^{-1})	4.8 (3.5 – 7.2)	5.4 (3.0 – 8.0)	5.0 (3.6 – 7.2)	0.021
LDL cholesterol (mmol l^{-1})	2.8 (1.6 – 4.5)	3.5 (1.7 – 5.6)	3.3 (1.9 – 4.9)	0.005*
HDL cholesterol (mmol l^{-1})	1.3 (0.8 – 2.1)	1.2 (0.7 – 3.5)	1.1 (0.7 – 1.8)	<0.001*
Fasting blood glucose (mmol l^{-1})	5.0 (4.3 – 7.1)	5.6 (4.7 – 7.0)	5.8 (4.4 – 14.7)	<0.001*
HbA1c (%)	5.5 (4.6 – 5.8)	5.4 (4.7 – 6.5)[a]	5.7 (4.8 – 9.5)[b]	0.001*
Free seminal L-carnitine (μg ml^{-1})	39.9 (8.6 – 168.3)	52.9 (8.9 – 158.9)	44.4 (10.8 – 326.3)	0.302
Total free seminal L-carnitine (μg per ejaculate)	184.3 (33.7 – 551.8)	138.8 (22.3 – 695.5)	181.5 (35.4 – 569.4)	0.898

*P≤0.01 were considered statistically significant; Kruskal–Wallis test was performed to examine the differences in the variables across BMI tertiles. [a]Missing/not reported values (n=1); [b]missing/not reported values (n=2). Variables are presented as median (range). BMI: body mass index; TG: triglyceride; LDL: low-density lipoprotein; HDL: high-density lipoprotein; HbA1c: glycated hemoglobin

Sperm motility was not assessed on the semen samples of the participants recruited from the fertility clinic.

Correlations between levels of free L-carnitine in seminal plasma and levels of sperm fatty acids

Sperm fatty acid composition is shown in **Table 2**. The two main fatty acids were palmitic acid (median: 24.6 wt%) and DHA (median: 23.4 wt%). The amount of free L-carnitine in the seminal plasma correlated positively with levels of sperm palmitic acid ($P = 0.014$), DHA ($P = 0.007$), and total n-3 PUFAs ($P = 0.008$) and negatively with lignoceric acid ($P = 0.001$) and total n-6 PUFAs ($P = 0.012$) when adjusted for covariates. After adjustment for possible covariates, the relation between the amount of eicosenoic acid and total PUFAs in spermatozoa and free seminal L-carnitine levels did not maintain statistical significance. Unadjusted levels of sperm DHA distributed by tertiles of total free L-carnitine in seminal plasma are shown in **Figure 1a**. Compared with the first tertile, sperm DHA levels were higher in the second and third tertile of total free L-carnitine distribution in the seminal plasma ($P < 0.001$).

Correlations between levels of free L-carnitine in seminal plasma, body mass, and semen parameter values

Levels of free L-carnitine in the seminal plasma were not correlated with BMI (**Table 3**). Unadjusted distribution of BMI in tertiles of free total L-carnitine in the seminal plasma is shown in **Figure 1b**. The amount of free L-carnitine in the seminal plasma correlated positively with age ($P = 0.013$) and sexual abstinence time ($P = 0.007$) in the unadjusted model (**Table 3**). Free seminal L-carnitine correlated positively with sperm concentration ($P < 0.001$), total sperm count ($P < 0.001$), progressive motility ($P = 0.002$), and MSC ($P < 0.001$), and negatively with semen volume ($P = 0.003$), when adjusted for covariates.

DISCUSSION

As far as we know, the relationship between levels of free seminal L-carnitine and sperm fatty acid content in humans has not been previously investigated. The composition of sperm fatty acid is important for semen quality. We and others have shown that sperm DHA is positively associated with sperm concentration, total count, progressive motility, vitality, and normal morphology.[5–7] In dog sperm, higher concentration of DHA is observed in cauda epididymis where mature spermatozoa are stored, than that in other epididymal segments.[38] L-carnitine is also found at high levels in the epididymal fluid,[23,24] and dietary supplementation with L-carnitine results in improved semen quality in infertile men,[39] as well as in increased levels of total n-3 PUFAs and improved semen quality in the rooster.[40] Here, we found that levels of free seminal L-carnitine correlated positively with the amount of total PUFAs, total n-3 PUFAs, palmitic acid, and DHA levels, and negatively with levels of total n-6 PUFAs and lignoceric acid in spermatozoa. Several of these sperm fatty acids have been previously shown to correlate with semen quality.[41] Our results underline the importance of sperm fatty acid composition in humans and suggest a potential specific role of seminal L-carnitine in sperm maturation in the epididymis.

Table 2: Associations between levels of free L-carnitine in seminal plasma (μg ml^{-1}) and levels of sperm fatty acids (n=128; wt%, of the total fatty acids analyzed by gas chromatography)

Variable	*Median (range)*	*Unadjusted*			*Adjusted*		
		B (95% CI)	*β*	*P*	*B (95% CI)*	*β*	*P*
Free seminal L-carnitine	44.4 (8.6 – 326.3)						
Myristic acid C14:0[a]	1.2 (0.5 – 2.9)	0.05 (–0.02 – 0.12)	0.13	0.134	0.06 (–0.01 – 0.13)	0.16	0.075
Palmitic acid C16:0[b]	24.6 (12.6 – 30.0)	112.06 (36.57 – 187.55)	0.25	0.004*	94.22 (19.41 – 169.02)	0.21	0.014*
Stearic acid C18:0	9.5 (6.7 – 13.9)	–0.43 (–1.12 – 0.27)	–0.11	0.226	–0.12 (–0.85 – 0.60)	–0.03	0.736
Arachidic acid C20:0[c]	0.64 (0.01 – 3.97)	–0.09 (–0.28 – 0.10)	–0.08	0.357	–0.06 (–0.26 – 0.15)	–0.05	0.585
Behenic acid C22:0	1.7 (0.1 – 3.7)	–0.40 (–0.81 – 0.01)	–0.17	0.056	–0.35 (–0.76 – 0.06)	–0.15	0.091
Lignoceric acid C24:0[a]	1.2 (0.1 – 8.5)	–0.27 (–0.41 – –0.13)	–0.32	<0.001*	–0.24 (–0.38 – –0.10)	–0.29	0.001*
SFAs total	38.8 (29.7 – 50.2)	0.71 (–1.44 – 2.86)	0.06	0.512	0.92 (–1.31 – 3.15)	0.08	0.414
Palmitoleic acid C16:1 n-7[c]	0.90 (0.04 – 3.27)	0.05 (–0.07 – 0.17)	0.08	0.398	0.09 (–0.03 – 0.20)	0.13	0.162
Oleic acid C18:1 n-9[c]	10.01 (0.01 – 29.54)	–0.42 (–0.89 – 0.06)	–0.15	0.089	–0.35 (–0.83 – 0.13)	–0.13	0.147
Eicosenoic acid C20:1 n-9[a]	0.4 (0.2 – 1.0)	–0.13 (–0.21 – –0.05)	–0.27	0.002*	–0.082 (–0.162 – –0.002)	–0.17	0.045
Nervonic acid C24:1 n-9[c]	1.7 (0.1 – 5.1)	–0.154 (–0.306 – –0.001)	–0.18	0.048	–0.187 (–0.324 – –0.004)	–0.20	0.044
MUFAs total[c]	13.0 (3.4 – 34.1)	–0.49 (–0.93 – –0.05)	–0.19	0.030	–0.41 (–0.83 – 0.02)	–0.16	0.061
LA C18:2 n-6[c]	4.0 (2.4 – 6.7)	–0.05 (–0.17 – 0.07)	–0.07	0.420	–0.07 (–0.19 – 0.06)	–0.09	0.309
ALA C18:3 n-3[c]	0.07 (0.02 – 0.57)	0.01 (–0.04 – 0.06)	0.04	0.656	0.02 (–0.04 – 0.07)	0.06	0.541
GLA C18:3 n-6[d]	0.03 (0.01 – 1.28)	12.12 (–2.58 – 26.83)	0.14	0.105	13.02 (–2.52 – 28.56)	0.15	0.100
EDA C20:2 n-6[c]	0.6 (0.2 – 1.3)	0.05 (–0.02 – 0.13)	0.13	0.146	0.04 (–0.03 – 0.11)	0.10	0.259
DGLA C20:3 n-6[a]	2.4 (0.9 – 8.2)	0.02 (–0.07 – 0.11)	0.04	0.628	0.03 (–0.07 – 0.12)	0.06	0.563
AA C20:4 n-6[a]	2.6 (1.0 – 8.4)	–0.084 (–0.171 – 0.003)	–0.17	0.058	–0.10 (–0.19 – –0.01)	–0.20	0.036
EPA C20:5 n-3[a]	0.07 (0.02 – 0.66)	–0.12 (–0.28 – 0.04)	–0.14	0.24	–0.19 (–0.36 – –0.03)	–0.21	0.024
DPA C22:5 n-3[c]	1.2 (0.5 – 2.7)	0.06 (–0.04 – 0.16)	0.10	0.265	0.01 (–0.01 – 0.11)	0.01	0.909
DHA C22:6 n-3[b]	23.4 (3.6 – 31.3)	229.81 (88.82 – 370.80)	0.28	0.002*	191.30 (52.66 – 329.93)	0.23	0.007*
PUFAs total[b]	35.51 (11.7 – 43.1)	337.45 (103.79 – 571.10)	0.25	0.005*	261.19 (38.59 – 483.79)	0.19	0.022
PUFAs n-3 total[b]	25.1 (4.9 – 33.1)	250.25 (96.96 – 403.54)	0.28	0.002*	204.23 (54.68 – 353.79)	0.23	0.008*
PUFAs n-6 total	10.0 (5.6 – 16.2)	–0.99 (–1.87 – –0.11)	–0.19	0.028	–1.21 (–2.15 – –0.28)	–0.24	0.012*

*$P \leq 0.01$ were considered statistically significant. Multiple linear regression analysis was used to determine associations between sperm fatty acids as dependent variables and free L-carnitine in seminal plasma as independent variable adjusted for age, sexual abstinence, and BMI. Free seminal L-carnitine and BMI were log-transformed. [a]Log-transformed variable; [b]squared-transformed variables; [c]square-root transformed variables; [d]inverse-transformed variable. Variables are presented as median (range). LA: linoleic acid; ALA: alpha-LA; GLA: gamma-LA; DGLA: dihomo-gamma-LA; EDA: eicosadienoic acid; AA: arachidonic acid: EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; SFAs: saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; B: regression coefficient; CI: confidence interval; β: standardized beta; wt%: weight percentage

Table 3: Associations between levels of free seminal L-carnitine (μg ml^{-1}; *n*=128) and semen parameter values

Variable	*n*	*Median (range)*	*Unadjusted*			*Adjusted*		
			B (95% CI)	*β*	*P*	*B (95% CI)*	*β*	*P*
Age (year)	128	36 (22 – 61)	6.68 (1.41 – 11.95)	0.22	0.013*			
BMI (kg m^{-2})[a]	128	28.4 (18.8 – 62.7)	0.002 (–0.06 – 0.06)	0.01	0.958			
Sexual abstinence (day)	125	3 (0 – 7)	1.20 (0.33 – 2.08)	0.24	0.007*			
Semen volume (ml)[b]	128	3.8 (1 – 10)	–0.35 (–0.58 – –0.11)	–0.25	0.004*	–0.37 (–0.60 – –0.13)	–0.27	0.003*
Sperm concentration (×10^6 ml^{-1})[b]	128	56 (1 – 350)	7.39 (5.62 – 9.17)	0.59	<0.001*	6.71 (4.85 – 8.57)	0.54	<0.001*
Total sperm count (×10^6)[b]	128	196 (6 – 1290)	11.56 (7.82 – 15.30)	0.48	<0.001*	10.49 (6.70 – 14.28)	0.43	<0.001*
Progressive motility (%)	93	43 (1 – 76)	18.98 (5.63 – 32.30)	0.28	0.006*	20.46 (7.69 – 33.24)	0.31	0.002*
Non-progressive motility (%)	93	27 (2 – 54)	–12.33 (–19.45 – –5.21)	–0.34	0.001*	–11.53 (–19.30 – –3.76)	–0.32	0.004*
MSC (×10^6 ml^{-1})[b]	93	49 (1 – 294)	5.66 (3.58 – 7.74)	0.49	<0.001*	5.02 (2.93 – 7.12)	0.44	<0.001*
Vitality (%)[b]	122	87 (40 – 97)	–0.42 (–0.81 – –0.04)	–0.19	0.032	–0.19 (–0.56 – 0.18)	–0.09	0.318
Normal forms (%)[b]	121	3 (0 – 11)	–0.16 (–0.59 – 0.26)	–0.07	0.450	–0.09 (–0.53 – 0.35)	–0.04	0.697
Head defects (%)[a]	121	97 (85 – 100)	0.80 (–1.19 – 2.79)	0.07	0.428	0.36 (–1.67 – 2.40)	0.03	0.724
Neck-mid-piece defects (%)[b]	121	25 (7 – 72)	–0.52 (–1.18 – 0.15)	–0.14	0.124	–0.39 (–1.11 – 0.33)	–0.11	0.283
Principal piece defects (%)[a]	121	13 (3 – 52)	–0.06 (–0.23 – 0.10)	–0.07	0.456	–0.18 (–0.33 – –0.02)	–0.19	0.026
DFI (%)[a]	99	15 (3 – 85)	0.14 (–0.02 – 0.31)	0.17	0.090	0.03 (–0.13 – 0.18)	0.03	0.747

*$P \leq 0.01$ were considered statistically significant. MSC was calculated by multiplying the sperm concentration and the percentage of motile spermatozoa divided by 100%. Multiple linear regression analysis was used to determine associations between semen parameter values as dependent variables and free L-carnitine in seminal plasma as independent variable adjusted for age, sexual abstinence, time to semen analysis and BMI. All variables were continuous except for time to semen analysis that was dichotomized (≤1 h or >1 h). Free seminal L-carnitine was log-transformed. [a]Log-transformed variables; [b]square-root transformed variables. Variables are presented as median (range). BMI: body mass index; MSC: motile sperm concentration; DFI: DNA fragmentation index; B: regression coefficient; CI: confidence interval; β: standardized beta

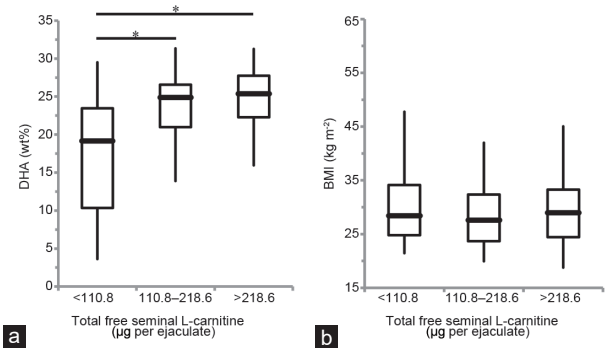

Figure 1: Sperm DHA levels and BMI by total free seminal L-carnitine distribution. Unadjusted sperm DHA levels in (**a**) weight percentage (wt%) and (**b**) BMI in relation to tertiles of total free seminal L-carnitine distribution in the study population ($n = 128$). Boxes represent interquartile range for 50% of the study population, with the middle line as median. Whiskers indicate minimum and maximum values, except for outliers that are not shown. Mann–Whitney U test was performed to examine the differences between the tertiles, *$P < 0.001$. DHA: docosapentaenoic acid; BMI: body mass index.

L-carnitine is known to have a central role in cellular aerobic metabolism by transporting long-chain fatty acids across the mitochondrial membranes for beta-oxidation.[10] Analysis of the sperm tail proteome showed that 24% of the proteins involved in metabolism and energy production consist of enzymes involved in lipid metabolism, including mitochondrial beta-oxidation and carnitine shuttle system.[34] In spermatozoa of several species, oxidation of fatty acids can provide the energy required for motility.[42,43] Accordingly, incubation of spermatozoa with etomoxir, an inhibitor of fatty acid oxidation, resulted in a significant decrease in sperm motility.[34] Furthermore, Banihani *et al*.[44] reported a significant increase in sperm motility *in vitro* in human semen samples supplemented with L-carnitine that could result from the involvement of L-carnitine in the beta-oxidation of fatty acids.

We found that levels of free seminal L-carnitine correlated with levels of sperm fatty acids important for sperm function. The mechanism underlying this association remains unknown. However, we cannot rule out a causal relationship between sperm fatty acid composition and free seminal L-carnitine levels. During transit of spermatozoa through the epididymis, sperm maturation involves remodeling of sperm membrane phospholipids with increase in DHA levels and in the ratio of PUFAs to SFAs in humans.[45] A knockout study in mice points toward an involvement of group III secreted phospholipase A2 (Pla2g3) expressed in epididymal epithelium in the rearrangement of fatty acids in spermatozoa during maturation.[46] In *Pla2g3*$^{-/-}$ mice, the sperm phospholipid composition showed higher SFA and lower n-3 PUFA levels compared to wild type mice, and sperm from *Pla2g3*$^{-/-}$ had impaired oocyte fertilization ability. Because of the changing fatty acid composition during maturation, fatty acids released from the spermatozoa membrane could function as signaling molecules in the epididymal epithelium to increase carnitine transport mediated by OCTN2 and OCTN3. Accordingly, palmitic acid upregulated the expression of *OCTN2* and *OCTN3* genes in the TM4 murine Sertoli cell line.[47] In addition, both human and animal *OCTN2* genes are regulated by the fatty acid-activated nuclear receptor, peroxisome proliferator-activated receptor alpha (PPARα), in hepatoma and kidney cell lines.[48] Our results showed a positive association between fatty acids important for sperm function and free seminal L-carnitine levels. This could be explained by a release of fatty acids from spermatozoa that can regulate L-carnitine transport from the bloodstream into the epididymal lumen.

In our study and as shown by others,[23,33] levels of free L-carnitine in the seminal plasma were correlated positively with sperm concentration, total count, and progressive motility and negatively with semen volume. In addition, we observed a positive relation between MSC and seminal L-carnitine levels. No correlation between free seminal L-carnitine and DFI was observed in our study, although supplementation with a combined formulation containing L-carnitine has previously been shown to reduce sperm DNA fragmentation.[49] In infertile men, administration of L-carnitine increased both sperm motility[50] and concentration.[51] The total oxyradical scavenging capacity of semen increased after L-carnitine supplementation[50] and rats that received L-carnitine intraperitoneally during testicular

gamma-irradiation showed a reduction in germ cell apoptosis.[52] Thus, L-carnitine could still have a positive effect on sperm concentration and total sperm count by its antioxidant and anti-apoptotic effects preserving membrane and DNA integrity of spermatozoa.

We have previously suggested that high BMI may be involved in the mechanism underlying reduced semen quality observed in obese men.[3] Since L-carnitine is crucial for normal lipid metabolism,[10] we investigated if there was a relation between free seminal L-carnitine levels and BMI but found no association. We have not found other studies that have investigated seminal L-carnitine in overweight or obese persons. Studies describing blood serum L-carnitine levels in relation to BMI show conflicting results[17–19] and are difficult to be compared with ours, as we did not measure L-carnitine in blood samples.

Strengths of this study are the broad BMI range of the study population and the fact that all semen samples were analyzed in the same laboratory according to the WHO standards. Moreover, none of the participants reported having consumed dietary supplements containing L-carnitine. Limitations of this study are that only levels of free, but not total or acetylated, L-carnitine were measured in seminal plasma and that no measurement of L-carnitine was performed in spermatozoa. Detailed analysis of the levels of these molecules could give more insights regarding the relationship between seminal L-carnitine and sperm fatty acids. In addition, seminal fatty acid content was not measured. The seminal fatty acid composition might have given an indication of the fatty acids of the epididymal epithelium. Another limitation of this study is that not all the semen samples were analyzed within 1 h after ejaculation.

It is worth noting that 18.8% of our participants were recruited at a fertility clinic. By using female infertility and not semen quality as inclusion criteria, we hypothesized that these men would reflect the general population and align with the rest of our study population. This was supported by our subanalysis. Significant differences between the two subgroups were seen in the length of sexual abstinence time and in normal sperm morphology. It is important to note that participants did not report their diet in this study and an influence of diet on the fatty acids' composition of spermatozoa and free seminal L-carnitine cannot be excluded.

In this study, we found that levels of free seminal L-carnitine positively correlated with the amount of palmitic acid, DHA, and total n-3 PUFAs of spermatozoa. Free seminal L-carnitine levels were not associated with BMI, indicating that reduced semen quality in obesity is independent of L-carnitine levels. Our findings are in line with the notion that the epididymis plays an active part in sperm maturation and that beta-oxidation might provide for energy production in mature spermatozoa. Further studies with causal research design are needed to investigate if free seminal L-carnitine may affect the fatty acid composition of the spermatozoa and if seminal L-carnitine is involved in energy production via fatty acids oxidation and oxidative phosphorylation in spermatozoa.

AUTHOR CONTRIBUTIONS

Conception of the study was initiated by MI. MI, OW, MHS, and TBH contributed to the study design. OW, MI, and JMA contributed substantially to the data collection. MI performed the statistical analysis. MI and OW drafted the manuscript. All authors contributed to interpretation of the data and critical revision of content. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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Supplementary Table 1: Overview of sperm fatty acids included in the dataset

Saturated fatty acids		*Monounsaturated fatty acids*		*Polyunsaturated fatty acids*	
Trivial name	*Symbol*	*Trivial name*	*Symbol*	*Trivial name*	*Symbol*
Myristic acid	C14:0	Palmitoleic acid	C16:1 n-7	LA	C18:2 n-6
Palmitic acid	C16:0	Oleic acid	C18:1 n-9	ALA	C18:3 n-3
Stearic acid	C18:0	Eicosenoic acid	C20:1 n-9	GLA	C18:3 n-6
Arachidic acid	C20:0	Nervonic acid	C24:1 n-9	EDA	C20:2 n-6
Behenic acid	C22:0			DGLA	C20:3 n-6
Lignoceric acid	C24:0			AA	C20:4 n-6
				EPA	C20:5 n-3
				DPA	C22:5 n-3
				DHA	C22:6 n-3

LA: linolenic acid; ALA: alpha-LA; GLA: gamma-LA; DGLA: dihomo-gamma-LA; EDA: Eicosadienoic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid

Supplementary Table 2: Comparison of free seminal L-carnitine levels between samples delivered within one hour after ejaculation or later

Variable	*≤1 h after ejaculation*		*>1 h after ejaculation*		*P*
	n	*Median (minimum-maximum)*	*n*	*Median (minimum-maximum)*	
Free seminal L-carnitine (μg mL^{-1})	104	45.7 (8.6–326.3)	24	36.4 (11.7–134.3)	0.204

P≤0.01 were considered statistically significant; Mann–Whitney *U* test was performed to compare the groups; Variables are presented as median with minimum and maximum values in brackets

Supplementary Table 3: Comparison of free seminal L-carnitine levels and semen parameter values between participants from the general population and from a fertility clinic

Variable	*General population*		*Fertility clinic***		*P*
	n	*Median (minimum–maximum)*	*n*	*Median (minimum–maximum)*	
Free seminal L-carnitine (μg mL^{-1})	104	44.2 (8.6–326.3)	24	41.4 (11.7–146.8)	0.475
BMI (kg m^{-2})	104	29.7 (18.8–62.7)	24	24 (20.7–27.1)	<0.001*
Age (years)	104	35 (22–61)	24	38 (35–51)	0.142
Sexual abstinence (days)#	101	3 (0–7)	24	3 (1–7)	0.004*
Semen volume (mL)	104	4 (1–10)	24	3 (2–6)	0.132
Sperm concentration (10^6 mL^{-1})	104	62 (3–350)	24	42 (1–222)	0.388
Total sperm count (10^6)	104	213 (6–1290)	24	146 (7–867)	0.241
Vitality (%)	104	87 (40–97)	18	88 (63–96)	0.726
Normal forms (%)	102	3 (0–10)	19	6 (2–11)	0.001*

*P≤0.01 were considered statistically significant; Mann–Whitney *U* test was performed to examine the differences in the variables between groups; **men from couples where female factor infertility was an inclusion criterion; not semen quality; #median values of sexual abstinence time in the two groups were the same, however; Mann–Whitney *U* test showed statistically significant differences between the groups; Variables are presented as median with minimum and maximum values in brackets. BMI: body mass index

Supplementary Table 4: Comparison of free seminal L-carnitine levels and semen parameter values between participants from the fertility clinic and from a subgroup of men from the general population with comparable body mass index (≤27 kg m^{-2})

Variable	*General population*		*Fertility clinic***		*P*
	n	*Median (minimum–maximum)*	*n*	*Median (minimum–maximum)*	
Free seminal L-carnitine (μg mL^{-1})	33	42.3 (8.6–168.3)	24	41.4 (11.7–146.8)	0.961
BMI (kg m^{-2})	33	23.7 (18.8–27.4)	24	24 (20.7–27.1)	0.599
Age (year)	33	29 (22–51)	24	38 (35–51)	<0.001*
Sexual abstinence (days)	33	4 (0–7)	24	3 (1–7)	0.008*
Semen volume (mL)	33	5 (2–8)	24	3 (2–6)	0.001*
Sperm concentration (10^6 mL^{-1})	33	70 (5–187)	24	42 (1–222)	0.210
Total sperm count (10^6)	33	345 (14–808)	24	146 (7–867)	0.021
Vitality (%)	33	90 (75–97)	18	88 (63–96)	0.119
Normal forms (%)	33	5 (0–10)	19	6 (2–11)	0.238

*P≤0.01 were considered statistically significant; Mann–Whitney *U* test was performed to examine the differences in the variables between groups; **men from couples where female factor infertility was an inclusion criterion; not semen quality; Variables are presented as median with minimum and maximum values in brackets. BMI: body mass index

Paper II

Association of endogenous seminal L-carnitine levels with post-thaw semen parameters in humans

Mario Iliceto, Mette Haug Stensen, Jorunn M. Andersen, Trine B. Haugen, Oliwia Witczak

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Research Article

Association of Endogenous Seminal L-Carnitine Levels with Post-Thaw Semen Parameters in Humans

Mario Iliceto,[1,2] **Jorunn M. Andersen**,[2] **Mette Haug Stensen**,[1] **Trine B. Haugen**,[2] **and Oliwia Witczak**[2]

[1]*Fertilitetssenteret, Oslo NO-0176, Norway*
[2]*Department of Life Sciences and Health, OsloMet-Oslo Metropolitan University, Oslo NO-0130, Norway*

Correspondence should be addressed to Mario Iliceto; marioi@oslomet.no and Oliwia Witczak; oliwiaw@oslomet.no

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Cryopreservation of semen is a useful tool for male fertility preservation. Some evidence for a beneficial effect of L-carnitine supplementation of freezing media on cryopreserved semen samples has been reported. Here, we examined the association of endogenous levels of seminal L-carnitine with post-thaw semen parameters. We also investigated the effect of freezing medium supplemented with L-carnitine on sperm characteristics, related to endogenous seminal L-carnitine levels. Semen analyses were performed on 125 fresh samples, and after standard cryopreservation and with L-carnitine as a supplement. Participants were categorized into two groups based on the median levels of endogenous seminal L-carnitine: low L-carnitine ($\leq$38.8 μg/ml) and high L-carnitine (>38.8 μg/ml). After standard cryopreservation, semen samples with high L-carnitine levels showed higher rapid progressive, progressive and total sperm motility and a reduced seminal static oxidation–reduction potential (ORP) level than samples with low L-carnitine levels. Only in post-thaw samples with low L-carnitine levels, there was an increase in the amount of sperm neck midpiece defects, compared to the fresh samples. Cryopreservation with L-carnitine had the most beneficial effect on rapid progressive sperm motility in samples with high endogenous L-carnitine levels. In conclusion, L-carnitine has a beneficial impact on sperm characteristics in post-thaw samples both as an endogenous component in seminal plasma and as a supplement in the freezing medium, by improving sperm motility and reducing seminal oxidative stress.

1. Introduction

Cryopreservation of semen in liquid nitrogen is a useful tool for male fertility preservation and is used routinely in assisted reproductive technology (ART) [1]. The procedure is used for donor sperm storage [2], prior to cancer treatments [3], in case of autoimmune diseases [4] and upon testicular and epididymal sperm extraction for ART [5]. Freezing and thawing have deleterious effects on sperm structure and function, including diminished sperm motility and viability [6–8] and increased DNA damage [6, 7, 9]. Several mechanisms causing cryopreservation-induced damage have been described, including cold shock, osmotic stress, intracellular ice crystal formation, and oxidative stress [10].

Oxidative stress is caused by an imbalance between the formation of reactive oxygen species (ROS) and antioxidant defenses [11]. Spermatozoa are highly susceptible to the deleterious effects of excessive ROS generation due to the high amount of polyunsaturated fatty acids (PUFAs) in the plasma membrane susceptible to lipid peroxidation [12]. Lipid peroxidation can lead to loss of membrane fluidity and integrity and can affect sperm structure and function [13, 14]. Increased levels of ROS in seminal plasma are associated with reduced sperm count [15] and sperm motility [16, 17] and increased DNA fragmentation [18].

Due to their small cytoplasmic volume, spermatozoa have limited amounts of ROS-scavenging enzymes. Therefore, the main antioxidant defense system of the spermatozoa is present in the seminal plasma [19, 20]. ROS-scavenging enzymes like glutathione peroxidase and glutathione transferase are found in both spermatozoa [21, 22] and seminal plasma [23, 24]. Enzymatic activities of superoxide dismutase (SOD) and catalase (CAT) have been measured in sperm [25, 26] and seminal

plasma [27]. Other important, nonenzymatic antioxidants present in seminal plasma are vitamins E and C [28–30].

L-carnitine, an amino acid derivative, plays an important role in fatty acids metabolism [31] and has been shown to reduce oxidative stress [32]. High amounts of L-carnitine originating from the epididymis are found in seminal plasma, and are positively associated with the total sperm count, sperm motility [33–35], sperm vitality, and sperm morphology [35] in humans. Supplementation of freezing media with L-carnitine has been shown to reduce cryopreservation-induced damage of the spermatozoa, improving post-thaw semen quality such as sperm motility [36–38] and sperm viability [37, 38], and reduce the amount of ROS in spermatozoa [39]. To our knowledge, no studies have investigated the association between endogenous levels of L-carnitine in seminal plasma and alterations in semen parameters upon freezing-thawing.

The aim of this study was to examine the association of endogenous levels of seminal L-carnitine with semen parameters after freezing-thawing. We also investigated the effect of freezing medium supplemented with L-carnitine on post-thaw semen parameters, related to the endogenous seminal L-carnitine levels.

2. Materials and Methods

2.1. Study Population. Men over the age of 18 years were recruited at the Fertilitetssenteret (Oslo, Norway), a fertility clinic that offers semen analysis for men. The recruitment was done between November 2018 and February 2020. The majority of the participants was not from couples undergoing IVF-treatment (89%). Incomplete samples, according to patient's information, were excluded from the study as well as samples with a sperm concentration less than 5×10^6/ml and/or with progressive motility of less than 10%. In total, 125 men were included in the study.

Participants reported information about sample collection, days of abstinence, weight, and height. Age was registered as whole years.

The study was approved by the Regional Committee for Medical and Health Research Ethics (REK), South East, Norway (REK number: 2018/423). All participants provided a written informed consent.

2.2. Semen Analysis. Semen samples were obtained by masturbation and collected on-site or at home in a preweighed sterile container. Participants were asked to abstain from ejaculating for 2–7 days before sample collection and to report the length of abstinence time. When collected at home, participants were instructed to avoid temperature variation of the sample during transportation. Out of 125 semen samples, 121 were collected on-site and four at home. Semen samples collected on-site were incubated for 30 min at 37°C, while samples collected at home were incubated for 10 min at 37°C. All of 125 semen samples were analyzed after complete liquefaction and within 1 hr after ejaculation. The ejaculated volume was estimated by weight. The methods recommended by the World Health Organization (WHO, 2010) [40] were employed to estimate sperm concentration, motility, and vitality. Sperm concentration was determined using an improved Neubauer hemocytometer (Hecht Assistant, Sondheim vor der Rhön, Germany). Sperm motility was categorized into progressive (Grades a + b), non-progressive (Grade c), and immotile sperm (Grade d). Total sperm motility was calculated (Grades a + b + c). In addition, rapid progressive motility was assessed (Grade a), which is a part of the categorization system in the WHO 1999 manual [41]. Vitality and morphology assessments were performed manually. In both fresh and post-thaw samples, 100 μl of semen was mixed with 500 μl Sperm Preparation Media (Cooper Surgical, Trumbull, CT, USA) and centrifugated at 300x g for 10 min. The supernatant was removed, and the pellet was resuspended in Sperm Preparation Media (Cooper Surgical, Trumbull, CT, USA) to obtain a sperm concentration of $5–10 \times 10^6$/ml. Vitality analysis was determined with BrightVit, a neosin and nigrosin staining solution (Microptic SL, Barcelona, Spain). Morphology was assessed according to the Tygerberg strict criteria [42], after Sperm Blue staining (Microptic SL, Barcelona, Spain). The semen analysis was performed by the same person, who participated in the external quality Program for semen assessment organized by ANOVA, Karolinska University Hospital, Sweden, when this study was conducted. Seminal plasma for L-carnitine analysis was prepared from the remaining ejaculated by centrifugation at 500x g for 15 min and stored at −80°C before analysis.

2.3. Cryopreservation of Spermatozoa. After initial semen analysis, two equal aliquots of each sample were prepared for cryopreservation: one for cryopreservation in standard sperm freezing medium (Cooper Surgical, Trumbull, CT, USA) and the other in the same sperm freezing medium supplemented with 0.5 mg/ml L-carnitine (Sigma Tau, Pomezia, Italy). To identify the optimal level for post-thaw sperm motility (Grades a + b + c), a gradient of L-carnitine concentrations (0, 0.1, 0.5, 1, 10, and 25 mg/ml) was added to the freezing medium of 10 semen samples. Figure S1 shows that 0.5 mg/ml L-carnitine added to the freezing medium had the best effect on post-thaw sperm motility. The selected value was consistent with the findings of a previous study [37]. Freezing medium was added dropwise to each aliquot, reaching a final dilution of 1 : 1. The mixture was left at room temperature for 20 min, then loaded into straws (Cryo bio system, Saint–Ouen Sur Iton, France) and sealed according to the manufacturer's recommendations. The straws were suspended horizontally above the surface of liquid nitrogen for 30 min, and then plunged into and stored in liquid nitrogen (−196°C). Before semen analysis, straws were thawed at room temperature for 5 min.

2.4. DNA Fragmentation. GoldCyto Sperm DNA kit (GC-SCD-20, GoldCyto Biotech, GuangZhou, China) was used to analyze DNA fragmentation according to the manufacturer's protocol for both fresh and cryopreserved samples. The method is based on the sperm chromatin dispersion (SCD) test [43, 44]. An aliquot of 100 μl semen sample was diluted in Sperm Preparation Media (Cooper Surgical, Trumbull, CT, USA) to obtain a sperm concentration of $5–10 \times 10^6$/ml. The cell suspension was then mixed with a low-melting-point agarose placed over an agarose pretreated slide

and left to solidify at 4°C for 5 min. The slides were immersed in a hydrochloric acid solution for 7 min at room temperature in the dark to generate restricted single-stranded DNA motifs from DNA breaks. After denaturation, nuclear proteins were removed using a lysing solution for 25 min at room temperature. The slides were placed into distilled water for 5 min, and dehydrated in sequential 70%, 90%, and 100% ethanol baths (2 min each) and air-dried. For staining, the slides were first immersed in TA solution (0.8 ml) for 1 min and then in TB solution (2.4 ml) for 6 min. The information about the staining ingredients is not available for the customers. To prevent the sediment from being deposited on the specimen, the slides were flushed with tap water, air dried and mounted with Eukitt (#253681, Panreac, Barcelona, Spain). The stained smears were examined under a bright light microscope (Olympus, Tokyo, Japan) at ×1,000 magnification. A minimum of 200 spermatozoa were counted. Spermatozoa with large or medium halos were defined as normal, whereas those with small or no halo and degraded spermatozoa were defined as fragmented. The SCD test results were expressed as % DNA fragmentation.

2.5. Seminal L-Carnitine Analysis. Level of L-carnitine (μg/ml) in free form in fresh seminal samples was analyzed by liquid chromatography/tandem mass spectrometry at a commercial laboratory in Oslo in 2020 (Vitas AS, Oslo, Norway) as previously described [33]. In brief, 10 μl of seminal plasma was diluted with labeled internal standard L-carnitine d9 (Sigma Aldrich, Taufkirchen, Germany) and analyzed on an Agilent 1260 HPLC coupled to an Agilent 6460 QQQ mass spectrometer operated in Multiple Reaction Monitoring Mode (Agilent Technologies, CA, USA).

2.6. Seminal Oxidation–Reduction Potential (ORP) Measurement. The ORP is the ratio of activity between oxidants and reductants and can be measured in biological samples such as blood plasma [45] and semen [46]. Static ORP (sORP) gives information about the current balance between oxidants and reductants/antioxidants in a sample, and has been confirmed to detect oxidative stress in blood plasma [45] and semen samples [47]. sORP was measured in millivolt (mV) in fresh and cryopreserved semen samples using the MiOXSYS platform (MiOXSYS, Aytu BioScience Inc, Englewood, CO, USA). 30 μl of semen was loaded into the MiOXSYS sensor and immediately inserted into the analyzer.

2.7. Statistical Analysis. All variables are presented as median with minimum and maximum values. Variables were evaluated for normal distribution using histogram, a quantile–quantile (Q–Q) plot and Kolmogorov–Smirnov test. For some of the participants ($n = 12$), body mass index (BMI) was not reported and for some of the fresh samples, data were lacking; sperm motility ($n = 15$), morphology ($n = 5$), and seminal sORP ($n = 1$). Data for post-thaw samples were lacking for sperm morphology ($n = 4$) and DNA fragmentation ($n = 1$). BMI was calculated as weight in kg divided by height in meters squared (kg/m^2).

Since, there is no established cutoff value for endogenous seminal L-carnitine, to detect differences in sperm parameters in relation to the endogenous levels of seminal L-carnitine, participants were categorized into two groups based on the 50th percentile (median) of L-carnitine levels: low L-carnitine ($\leq$38.8 μg/ml) and high L-carnitine (>38.8 μg/ml). Mann–Whitney *U* test was used to compare differences between the two groups. Wilcoxon signed-rank test was used to compare semen variables in samples after standard cryopreservation and after L-carnitine supplementation.

Absolute change in semen quality values was used to examine the difference between L-carnitine supplementation and standard cryopreservation by subtraction of the standard cryopreservation median value from the supplemented cryopreservation median value for each parameter. Mann–Whitney *U* test was used to compare variables between the groups.

Box plots were used to visualize the effect of various L-carnitine concentrations supplemented to the freezing medium on post-thaw total sperm motility, and Wilcoxon signed-rank test was used to compare the difference between the groups. Jitter plots were used to visualize the distribution of sperm motility in fresh samples, after standard cryopreservation and after L-carnitine supplementation, as well as the difference in sperm motility between samples with low and high L-carnitine levels in seminal plasma. Mann–Whitney *U* test was used to compare variables between the groups.

The distribution of endogenous seminal L-carnitine levels is presented as a histogram plot. Variable distributions were evaluated by histograms. Skewed variables were log-, square-root, squared-, or cubic-transformed. Pearson's correlation coefficient was used to measure associations between levels of seminal L-carnitine and semen characteristics. Pearson's partial correlation coefficient was used to control for semen volume in addition. All variables were continuous. Associations between levels of seminal L-carnitine and semen characteristics are presented as unadjusted and adjusted for semen volume.

Statistical analyses were performed by using IBM SPSS Statistics 20 (IBM, Chicago, IL, USA). To minimize the risk of type I errors due to multiple comparisons, the level of statistical significance was set at p-value ≤ 0.01.

3. Results

3.1. Study Population. The characteristics of the study population are presented in Table 1. When comparing the semen analysis results with lower reference limits defined by the WHO (2010) [40], a total of 21 men had a sperm concentration below 15×10^6/ml, and 11 men had a total sperm count below 39×10^6/ml. Regarding motility, 55 men had <25% of rapid progressive sperm, 30 had <32% of progressive motile sperm and 26 had <40% total motile sperm.

Endogenous seminal L-carnitine levels were positively associated with sperm concentration, total sperm count as well as rapid progressive and progressive motility (Table 1), as in agreement with previous studies [33–35].

The distribution of endogenous seminal L-carnitine in seminal plasma is shown in Figure 1. The mean (SD) value of endogenous seminal L-carnitine was 48.5 $\pm$ 32.7 μg/ml.

3.2. Semen Parameters in Fresh and Post-Thaw Samples with Low and High Endogenous Seminal L-Carnitine Levels. To examine the relation between endogenous levels of seminal

Table 1: Correlations between levels of endogenous seminal L-carnitine ($n = 125$; μg/ml) and background characteristic values.

Variable	n	Median (min–max)	Unadjusted		Adjusted for semen volume	
			r	p	r	p
Age (years)[a]	125	35 (23–57)	0.013	0.895	−0.062	0.531
BMI (kg/m^2)[b]	113	24.7 (17.4–40.2)	−0.116	0.221	−0.029	0.761
Sexual abstinence (days)[b]	125	3 (1–7)	0.069	0.484	0.139	0.158
Semen volume (ml)[b]	125	4 (1–8)	−0.415	<0.001*	—	
Sperm concentration (10^6/ml)[b]	125	38 (5–239)	0.695	<0.001*	0.672	<0.001*
Total sperm count (10^6)[b]	125	126 (14–521)	0.496	<0.001*	0.672	<0.001*
Rapid progressive sperm motility (a, %)	110	25 (2–55)	0.356	<0.001*	0.415	<0.001*
Progressive sperm motility (a + b, %)	110	40 (11–63)	0.238	0.014*	0.295	0.002*
Total sperm motility (a + b + c, %)	110	51 (18–74)	0.178	0.067	0.246	0.011*
Immotile sperm (d, %)[b]	110	50 (26–82)	−0.178	0.069	−0.246	0.011*
Sperm vitality (%)[c]	125	61 (19–88)	0.194	0.047	0.210	0.032
Normal sperm morphology (%)[b]	120	8 (1–32)	0.099	0.311	0.089	0.368
Sperm head defects (%)[d]	120	88 (56–99)	−0.076	0.437	−0.083	0.403
Spem neck midpiece defects (%)	120	28 (3–55)	−0.058	0.554	−0.091	0.358
Sperm principal piece defects (%)[b]	120	12 (3–31)	−0.160	0.102	−0.145	0.139
Fragmented sperm DNA (%)[a]	125	15 (2–88)	−0.150	0.126	−0.189	0.054
Seminal sORP (mV)[b]	124	41 (3–172)	0.004	0.965	0.046	0.640

BMI, body mass index; sORP, static oxidation-reduction potential; r, correlation coefficient. *p values ≤ 0.01 were considered statistically significant. Pearson's correlation analysis was used to determine correlation between levels of endogenous L-carnitine in seminal plasma and semen parameters. Pearson's partial correlation analysis was used for adjusted data. Data are presented as unadjusted and adjusted for semen volume. All variables were continuous. [a]Log-transformed variables. [b]Square-root transformed variables. [c]Power transformed variables. [d]Cubic transformed variables.

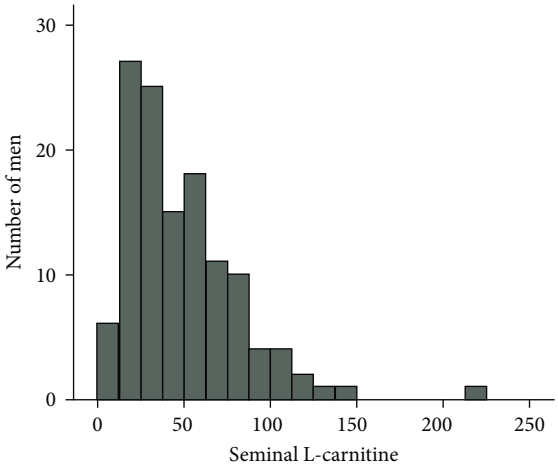

Figure 1: Distribution of L-carnitine levels (μg/ml) in seminal plasma in the study population ($n = 125$).

L-carnitine and semen quality parameters before and after cryopreservation, the population was categorized into two groups according to the median L-carnitine value (38.8 μg/ml) and classified either to low (≤38.8 μg/ml) or high (>38.8 μg/ml) endogenous seminal L-carnitine group. There was no difference in BMI, age nor the time of abstinence between the two groups. In fresh samples, the percentage of rapid progressive sperm was higher in the group with high seminal L-carnitine levels than in the group with low levels (Table 2). After standard cryopreservation, spermatozoa in samples with high L-carnitine levels had higher rapid progressive, progressive, and total sperm motility than spermatozoa in samples with low L-carnitine levels (Table 2). In post-thaw samples, seminal sORP level was lower in the group with high L-carnitine levels than the comparing group, while sperm vitality, morphology and DNA fragmentation did not differ between the two groups. When comparing semen quality variables in fresh versus post-thaw samples, the levels of sperm neck midpiece defects after cryopreservation were higher in the group with low L-carnitine levels, while there was no difference in the group with high L-carnitine levels. No other differences in semen quality parameters were observed between fresh and post-thaw samples.

Sperm motility in fresh samples and after cryopreservation, according to the low and high seminal L-carnitine groups are presented in Figure S2.

3.3. Effect of L-Carnitine Supplementation on Post-Thaw Semen Parameters in Samples with Low and High Endogenous Seminal L-Carnitine Levels. L-carnitine supplementation of the freezing medium had a beneficial effect on semen quality after freezing and thawing (Table S1). Both groups showed higher proportions of rapid progressive sperm motility, progressive motility, total motility and vitality compared to standard cryopreservation (Table 3). Seminal sORP levels were lower after L-carnitine supplementation in both groups compared to standard cryopreservation. No impact was seen on sperm morphology parameters after cryopreservation with L-carnitine. Distribution of sperm motility after cryopreservation with L-carnitine in samples with low and high endogenous seminal L-carnitine levels is visualized in Figure S2. To sum up, L-carnitine supplementation has a positive effect on post-thaw semen parameters in samples with both high and low endogenous L-carnitine.

Table 2: Semen parameters in fresh and post-thaw samples according to high and low endogenous seminal L-carnitine levels.

Variable	Fresh samples					Post-thaw samples					Fresh vs post-thaw samples	
	Low seminal L-carnitine levels[#] (≤38.8 μg/ml)		High seminal L-carnitine levels[#] (>38.8 μg/ml)			Low seminal L-carnitine levels[#] (≤38.8 μg/ml)		High seminal L-carnitine levels[#] (>38.8 μg/ml)			Low seminal L-carnitine levels[#] (≤38.8 μg/ml)	High seminal L-carnitine levels[#] (>38.8 μg/ml)
	n	Median (min–max)	*n*	Median (min–max)	*p*	*n*	Median (min–max)	*n*	Median (min–max)	*p*	*p*	*p*
Endogenous seminal L-carnitine (μg/ml)	63	25 (6–38.8)	62	68 (39–220)	<0.001*	—	—	—	—	—	—	—
Semen volume (ml)	63	4 (2–8)	62	3 (1–7)	0.003*	—	—	—	—	—	—	—
Sperm concentration (10^6/ml)	63	23 (5–73)	62	56 (11–239)	<0.001*	—	—	—	—	—	—	—
Total sperm count (10^6)	63	81 (14–413)	62	187 (25–521)	<0.001*	—	—	—	—	—	—	—
Rapid progressive sperm motility (a, %)	53	17 (2–42)	57	28 (4–55)	0.002*	63	7 (0–27)	62	12 (2–26)	0.002*	<0.001*	<0.001*
Progressive sperm motility (a + b, %)	53	35 (11–59)	57	43 (15–63)	0.037	63	17 (0–39)	62	25 (3–40)	0.002*	<0.001*	<0.001*
Total sperm motility (a + b + c, %)	53	48 (18–70)	57	54 (21–74)	0.113	63	27 (1–51)	62	36 (5–51)	<0.001*	<0.001*	<0.001*
Immotile sperm (d, %)	53	52 (30–82)	57	46 (26–79)	0.113	63	73 (49–99)	62	65 (49–95)	<0.001*	<0.001*	<0.001*
Sperm vitality (%)	63	57 (19–88)	62	63 (30–80)	0.027	63	34 (4–53)	62	38 (16–58)	0.051	<0.001*	<0.001*
Normal sperm morphology (%)	61	8 (1–31)	59	8 (1–32)	0.771	61	7 (1–29)	60	7 (0–24)	0.862	0.510	0.032
Sperm head defects (%)	61	88 (66–96)	59	87 (56–99)	0.429	61	88 (59–99)	60	86 (72–99)	0.134	0.648	0.789
Sperm neck midpiece defects (%)	61	28 (9–55)	59	26 (3–55)	0.825	61	32 (3–67)	60	30 (4–61)	0.220	0.008*	0.127
Sperm principal piece defects (%)	61	13 (3–31)	59	12 (3–26)	0.676	61	15 (5–37)	60	14 (6–36)	0.213	<0.001*	0.003*
Sperm DNA fragmentation (%)	63	15 (6–88)	62	14 (2–54)	0.204	62	22 (8–62)	62	20 (6–48)	0.192	0.002*	<0.001
Seminal sORP (mV)	63	41 (3–172)	61	41 (3–97)	0.601	62	66 (21–118)	62	55 (19–146)	0.005*	<0.001*	<0.001*

[#]The population was categorized in two groups according to the 50th percentile of endogenous seminal L-carnitine levels. sORP, static oxidation-reduction potential. *p values ≤ 0.01 were considered statistically significant. Mann–Whitney *U* test was used to compare variables in samples with low and high seminal L-carnitine levels. Wilcoxon signed-rank test was used to compare semen quality variables in fresh and post-thaw samples.

Table 3: Semen parameters in samples with high and low endogenous seminal L-carnitine levels after standard cryopreservation and cryopreservation supplemented with L-carnitine.

Variable	Low seminal L-carnitine levels[#] (≤38.8 μg/ml)					High seminal L-carnitine levels[#] (>38.8 μg/ml)				
	Cryopreservation method									
	Standard		Supplemented with L-carnitine			Standard		Supplemented with L-carnitine		
	n	Median (min–max)	*n*	Median (min–max)	*p*	*n*	Median (min–max)	*n*	Median (min–max)	*p*
Rapid progressive sperm motility (a, %)	63	7 (0–27)	63	11 (1–32)	<0.001*	62	12 (2–26)	62	18 (2–37)	<0.001*
Progressive sperm motility (a + b, %)	63	17 (0–39)	63	21 (2–44)	<0.001*	62	25 (3–40)	62	30 (5–50)	<0.001*
Total sperm motility (a + b + c, %)	63	27 (1–51)	63	33 (3–54)	<0.001*	62	36 (5–51)	62	41 (7–56)	<0.001*
Immotile sperm (d, %)	63	73 (49–99)	63	67 (46–97)	<0.001*	62	65 (49–95)	62	60 (44–93)	<0.001*
Sperm vitality (%)	63	34 (4–53)	63	35 (11–60)	0.003*	62	38 (16–58)	62	43 (16–55)	<0.001*
Normal sperm morphology (%)	61	7 (1–29)	61	7 (2–26)	0.099	60	7 (0–24)	60	7 (0–26)	0.302
Sperm head defects (%)	61	88 (59–99)	61	88 (65–98)	0.738	60	86 (72–99)	60	86 (66–99)	0.598
Sperm neck midpiece defects (%)	61	32 (3–67)	61	31 (13–61)	0.386	60	30 (4–61)	60	29 (14–64)	0.772
Sperm principal piece defects (%)	61	15 (5–37)	61	14 (5–33)	0.203	60	14 (6–36)	60	13 (1–37)	0.282
Sperm DNA fragmentation (%)	62	22 (8–62)	62	20 (6–64)	0.032	62	20 (6–48)	62	19 (5–47)	0.017
Seminal sORP (mV)	62	66 (21–118)	62	64 (18–117)	0.004*	62	55 (19–146)	62	51 (14–132)	0.007*

[#] The population was categorized in two groups according to the 50[th] percentile of endogenous seminal L-carnitine levels. sORP, static oxidation-reduction potential. *p values ≤ 0.01 were considered statistically significant. Wilcoxon signed-rank test was used to compare post-thaw semen variables between standard and supplemented with L-carnitine.

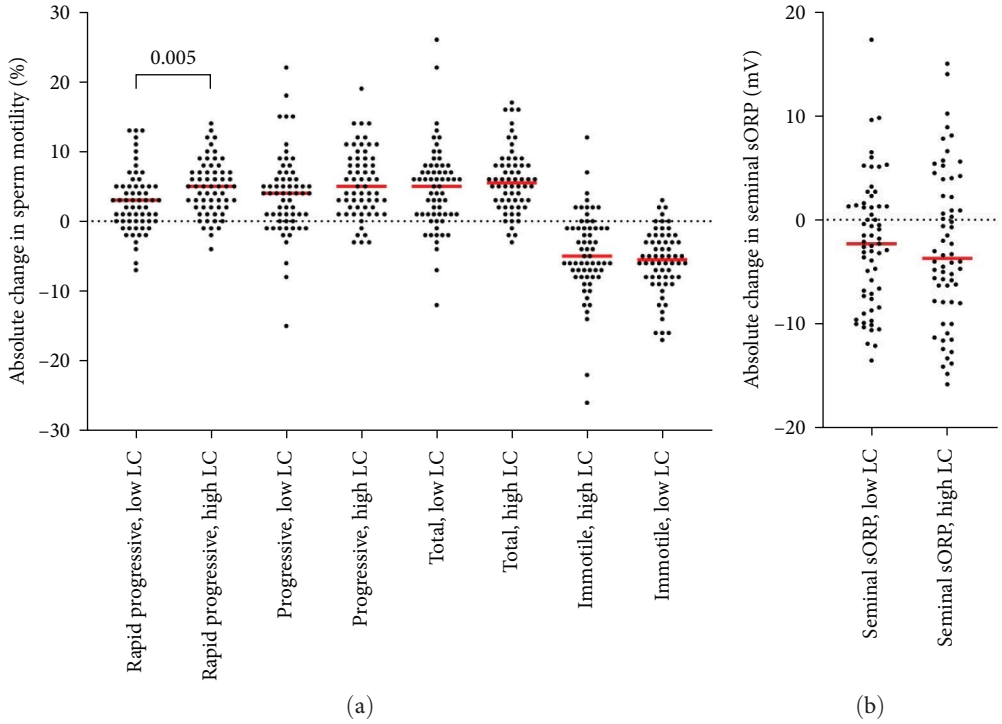

FIGURE 2: Absolute change in sperm motility and seminal sORP levels between post-thaw samples supplemented with L-carnitine and after standard cryopreservation in groups with low and high seminal L-carnitine. Absolute change in sperm motility (a) and seminal sORP levels (b) between post-thaw samples supplemented with L-carnitine and after standard cryopreservation in groups with low LC ($\leq$38.8 μg/ml, $n =$ 63 for motility and $n = 62$ for seminal sORP) and high LC (>38.8 μg/ml, $n = 62$ for both) levels. Every point in the jitter plot represents an absolute change. Horizontal lines for each group represent median, while the dotted line at 0 indicates no change. Mann–Whitney U test was used to compare differences between the groups. p values ≤ 0.01 were considered statistically significant. sORP: static oxidation-reduction potential; LC: L-carnitine.

To further examine potential differences in the impact of L-carnitine supplementation on semen parameters between samples with high and low endogenous L-carnitine levels, we quantitated the absolute change in post-thaw semen parameters by subtracting the standard cryopreservation median value from the supplemented cryopreservation median value for each parameter in the two groups. For progressive sperm motility, total motility, the proportion of immotile sperm and sORP there were no differences in the improvement by L-carnitine supplementation between the two groups. (Figures 2(a) and 2(b)). Neither were there differences in improvement in sperm DNA fragmentation, and normal sperm morphology between the two groups after L-carnitine supplementation (Table S2). However, the improvement in rapid progressive sperm motility after L-carnitine supplementation was highest in the group with high endogenous L-carnitine levels and statistically significant compared to the low L-carnitine group (Figure 2(a) and Table S2).

4. Discussion

The impact of endogenous seminal L-carnitine levels on post-thaw semen quality has not been investigated although a beneficial effect of L-carnitine supplementation of freezing medium has been shown [37, 38]. Here, we show that the amount of post-thaw motile sperm was higher in the group of semen samples with high endogenous seminal L-carnitine levels, compared to the group with low levels. This association does not imply causality but indicates that the harmful effects of cryopreservation are reduced in samples with high levels of L-carnitine in seminal plasma. There was no difference in post-thaw sperm morphology, vitality, or DNA fragmentation between the two L-carnitine groups. We also show an oxidative stress increase in post-thaw samples in both low and high endogenous seminal L-carnitine groups, as indicated by increased levels of seminal sORP compared to fresh samples (Table 2). However, levels of seminal sORP increased less after cryopreservation when endogenous seminal L-carnitine levels were high. This result is in line with the reported ability of L-carnitine to prevent oxidative stress [48–50].

In addition to increased oxidative stress in post-thaw samples, cryopreservation is associated with sperm mitochondrial dysfunction, lipid peroxidation, reduced sperm motility and morphology [6, 8, 51]. O'Connell et al. [8] demonstrated that mitochondrial dysfunction measured by accumulation of rhodamine 123 (R123) into spermatozoa was positively related to the level of sperm midpiece defects. A functional sperm midpiece is important for optimal sperm motility [52], as energy for sperm movement is derived from mitochondria located in the midpiece [53]. Mitochondrial function was not measured in this study. However, we show an increase in the amount of sperm neck midpiece defects only in post-thaw

samples with low endogenous seminal L-carnitine levels, compared to fresh samples. In fresh semen samples, there was no difference in sperm neck and midpiece defects between samples with low and high L-carnitine levels, which may indicate a protective impact of endogenous L-carnitine against damage sustained during cryopreservation. In addition, the proportion of spermatozoa with progressive and total motility was higher, and there were less immotile sperm in post-thaw samples with high levels of endogenous L-carnitine. Concomitantly, seminal sORP levels were lower in these samples. Several studies have shown association between increased oxidative stress and reduced sperm motility due to lipid peroxidation [17, 54, 55]. Evidence indicates that compounds with anti-oxidant properties can improve sperm motility in mammalian spermatozoa by suppressing lipid peroxidation [56–58]. Increased lipid peroxidation has been associated with cryopreservation in mammalian spermatozoa [51, 59–61]. We did not measure lipid peroxidation in this study. Overall, our results support a role for endogenous seminal L-carnitine in reducing oxidative stress in post-thaw samples.

It is worth mentioning that no relation between endogenous seminal L-carnitine levels and seminal sORP levels nor sperm DNA fragmentation index (DFI) was observed in fresh samples (Table 2). However, both high endogenous levels of L-carnitine and supplementation of the freezing medium with L-carnitine were beneficial for semen quality in post-thaw samples when the level of oxidative stress increased.

In our study and as well as in others' [37, 38], L-carnitine supplementation of the freezing medium was beneficial for post-thaw semen quality. In addition, we show here that the beneficial effect on rapid progressive sperm motility observed after cryopreservation with L-carnitine was higher in samples with high endogenous seminal L-carnitine levels compared to those with low endogenous levels. Since, there was no difference in change in oxidative stress levels between low and high endogenous L-carnitine groups when comparing the two cryopreservation protocols, one can presume that the higher presence of sperm with rapid motility may result from the well-characterized role of L-carnitine in energetic metabolism [31]. Since, L-carnitine is required for transport of long-chain fatty acids into mitochondria for β-oxidation and production of ATP, it is likely that the improvement in sperm motility was due to increased delivery of energy substrates into the mitochondria. This is in agreement with a previous *in vitro* study showing that incubation of semen samples with L-carnitine increased sperm motility, but in this study a longer timepoint for analysis compared to ours was used [62]. In addition, global DNA methylation levels are positively associated with motility in human spermatozoa [63]. A recent study has shown that global methylation decreases after cryopreservation in buck sperm and that L-carnitine supplementation of the freezing medium reversed the decrease in levels of DNA methylation [64]. As there is a negative correlation between ROS levels and global sperm DNA methylation [65], one can hypothesize that the antioxidant properties of L-carnitine will contribute to a protecting environment for global DNA methylation in post-thaw spermatozoa with high endogenous and/or supplemented levels.

The effect of L-carnitine supplementation on post-thaw sperm motility (a, a + b, a + b + c grades) was not beneficial for all samples compared to standard cryopreservation. Furthermore, samples with low endogenous L-carnitine levels showed a larger distribution of absolute changes in sperm motility grades compared to samples with high endogenous L-carnitine levels. This suggests that the post-thaw outcome of L-carnitine supplementation varies more in samples with low endogenous L-carnitine levels. Cryopreservation has been shown to reduce seminal SOD activity in an inter-individual manner, with large variations between donor samples [51]. This was associated with a decrease in sperm phospholipid content due to lipid peroxidation. As mentioned earlier, we did not measure lipid peroxidation, but our results support the notion that individual semen samples are differently affected by cryopreservation.

A strength of the study is that semen analysis was performed by a person participating in the external quality program for semen assessment. The semen samples collected in the study represented a broad range of semen quality values. A limitation of the study is that only one concentration of L-carnitine was used in the freezing medium, where total motility was the only outcome that was evaluated. Furthermore, in this study post-thaw sperm motility was assessed 5 min after thawing, according to the manufacturers protocol. The sperm motility tested after a longer post-thaw period could have given other results, as it has been shown that sperm motility increases with time [66].

In conclusion, L-carnitine has a beneficial impact on semen quality in post-thaw samples both as an endogenous component in seminal plasma and as a supplement in the freezing medium, by improving sperm motility and diminishing seminal oxidative stress. We also observed low levels of sperm neck midpiece defects after cryopreservation in samples with high endogenous L-carnitine levels. Altogether, these findings indicate that high levels of L-carnitine in seminal plasma are protective against the harmful effect of cryopreservation.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

All authors declare that they have no conflicts of Interest.

Authors' Contributions

Conception of the study was initiated by OW. MI, TBH, JMA, and MHS contributed to the study design. MI collected the data and performed the statistical analyses. Visualization of the data was done by OW and MI. OW and MI drafted the manuscript. All authors contributed to interpretation of the data and critical revision of content. All authors read and approved the final manuscript.

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Supplementary Materials

Figure S1: describes the effect of L-carnitine concentrations supplemented to the freezing media on post-thaw total sperm motility. Table S1: describes the comparison of semen parameters after standard cryopreservation and supplemented with L-carnitine (0.5 mg/ml). Table S2: describes the absolute change (Δ) in post-thaw semen variables between cryopreservation supplemented with L-carnitine and standard cryopreservation in semen samples with low and high endogenous L-carnitine (LC) levels. Figure S2: sperm motility in fresh samples, after standard cryopreservation, and after cryopreservation supplemented with L-carnitine categorized according to endogenous L-carnitine levels. (*Supplementary Materials*)

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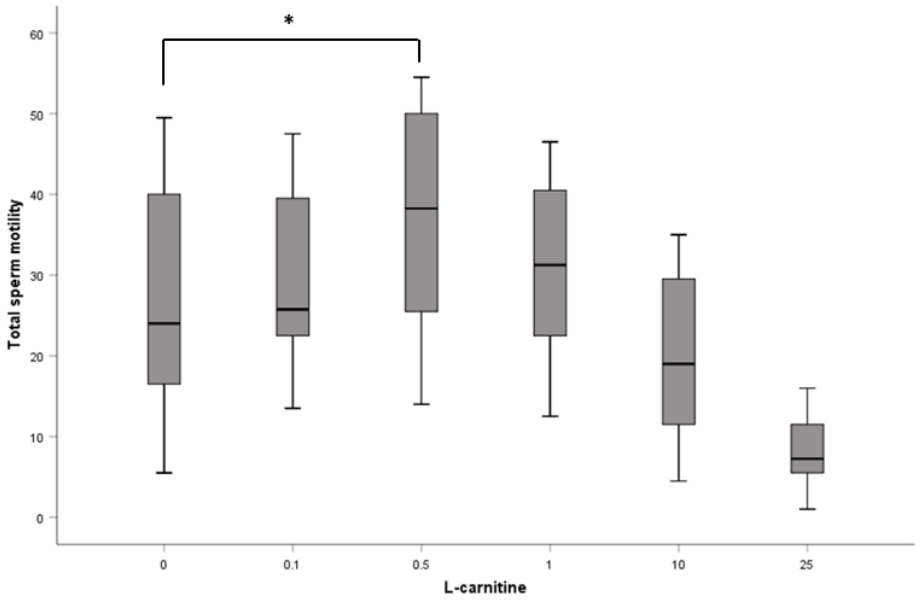
Supplementary information



Supplementary Figure S1. Effect of L-carnitine concentrations supplemented to the freezing media on post-thaw total sperm motility. Effect of various L-carnitine concentrations (mg/ml) supplemented to the freezing medium on post-thaw total sperm motility (n = 10). Box plots represents the distribution of total sperm motility (grade a + b + c) in percent. Horizontal lines for each group represent median. Whiskers indicate minimum and maximum values. Wilcoxon signed-rank test was used to compare the difference between the groups. * *p* value < 0.01.

Supplementary Table S1. Comparison of semen parameters after standard cryopreservation and supplemented with L-carnitine (0.5 mg/ml)

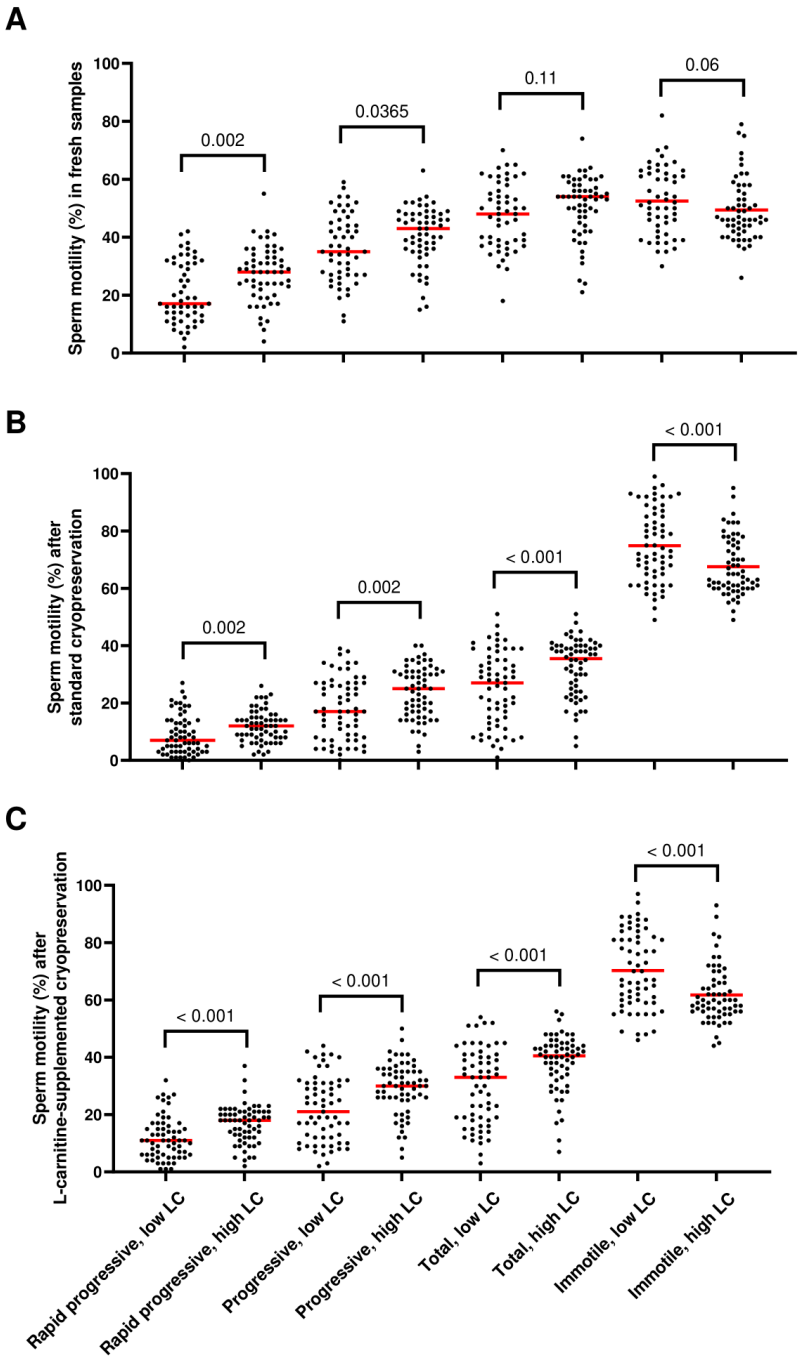
		Cryopreservation		
		Standard	Supplemented with L-carnitine	
Variable	***n***	**Median (min – max)**	**Median (min – max)**	***p***
Rapid progressive sperm motility (a, %)	125	10 (0 – 27)	14 (1 – 37)	< 0.001*
Progressive sperm motility (a + b, %)	125	21 (0 – 40)	27 (2 – 50)	< 0.001*
Total sperm motility (a + b + c, %)	125	31 (1 – 51)	38 (3 – 56)	< 0.001*
Immotile sperm (d, %)	125	69 (49 – 99)	62 (44 – 97)	< 0.001*
Sperm vitality (%)	125	35 (40 – 58)	40 (11 – 60)	< 0.001*
Normal sperm morphology (%)	121	7 (0 – 29)	7 (0 – 26)	0.054
Sperm head defects (%)	121	86 (59 – 99)	86 (65 – 69)	0.551
Sperm neck midpiece defects (%)	121	31 (3 – 67)	30 (13 – 64)	0.740
Sperm principal piece defects (%)	121	15 (5 – 37)	13 (1 – 37)	0.100
Fragmented sperm DNA (%)	124	21 (6 – 62)	19 (5 – 64)	0.002*
Seminal sORP (mV)	124	60 (19 – 146)	56 (14 – 132)	< 0.001*

sORP, static oxidation-reduction potential. * *p* values ≤0.01 were considered statistically significant. Wilcoxon signed-rank test was used to compare variables between semen samples frozen with and without L-carnitine.

Supplementary Table S2. Absolute change (Δ) in post-thaw semen variables between cryopreservation supplemented with L-carnitine and standard cryopreservation in semen samples with low and high endogenous L-carnitine (LC) levels.

	Low seminal LC levels[#] (≤ 38.8 µg/ml)		High seminal LC levels[#] (> 38.8 µg/ml)		
Variable	***n***	**Median (min – max)**	***n***	**Median (min – max)**	***p***
Δ rapid progressive sperm motility (a)	63	3 (-7 – 13)	62	5 (-4 – 14)	0.005*
Δ progressive sperm motility (a + b)	63	4 (-15 – 22)	62	5 (-3 – 19)	0.074
Δ total sperm motility (a + b + c)	63	5 (-12 – 26)	62	6 (-3 – 17)	0.224
Δ sperm vitality	63	2 (-22 – 31)	62	4 (-5 – 19)	0.244
Δ sperm neck midpiece defects	61	0 (-21 – 22)	60	0 (-9 – 31)	0.256
Δ sperm DNA fragmentation	62	-1 (-13 – 8)	62	-2 (-12 – 8)	0.896
Δ seminal sORP	62	-2 (-14 – 17)	62	-4 (-16 – 15)	0.608

[#] The population was categorized in two groups according to the 50th percentile of endogenous seminal L-carnitine levels. sORP, oxidation-reduction potential. * *p* values ≤0.01 were considered statistically significant. Mann-Whitney U test was used to compare the two groups.



Supplementary Figure S2. Sperm motility in fresh samples, after standard cryopreservation, and after cryopreservation supplemented with L-carnitine categorized according to endogenous L-carnitine levels. Sperm motility in fresh samples (A), after standard cryopreservation (B), and after cryopreservation supplemented with L-carnitine (C) categorized according to endogenous L-carnitine levels: low LC (≤ 38.8 µg/ml, $n = 63$) and high LC (> 38.8 µg/ml, $n = 62$). Jitter plots represents the distribution of sperm motility in percent. Horizontal lines for each group represent median. Mann-Whitney U test was used to compare the difference between the groups. p values ≤ 0.01 were considered statistically significant. LC: L-carnitine.

Paper III

Assessment of alternative methods to Sperm Chromatin Structure Assay for evaluating DNA damage induced by oxidative stress in bull sperm

Ingvild Aas, Mario Iliceto, Else-Berit Stenseth, Anne Hege Alm-Kristiansen, Oliwia Witczak, and Erwan Delbarre

Manuscript

Assessment of alternative methods to Sperm Chromatin Structure Assay for evaluating DNA damage induced by oxidative stress in bull sperm

Ingvild Aas [1], Mario Iliceto [1, 2], Anne Hege Alm-Kristiansen [3], Else-Berit Stenseth [4], Oliwia Witczak [1,*], and Erwan Delbarre [3,*]

[1] Department of Life Sciences and Health, Faculty of Health Sciences, OsloMet – Oslo Metropolitan University, Oslo, Norway

[2] Volvat Spiren, Oslo, Norway

[3] CRESCO, Centre for Embryology and Healthy Development, Department of Biotechnology, Inland Norway University of Applied Sciences, Hamar, Norway.

[4] Department of Biotechnology, Inland Norway University of Applied Sciences, Hamar, Norway.

* Correspondence: ED: Erwan.delbarre@inn.no; OW: oliwiaw@oslomet.no

Abstract

DNA damage in mammalian spermatozoa is associated with reduced fertilizing capacity and has been shown to negatively impact fetal development in offspring. Sperm Chromatin Structure Assay (SCSA®) is considered a gold standard for measuring sperm DNA integrity, including the presence of single-strand and/or double-strand breaks. However, its expensive implementation deters many fertility clinics and breeding companies from using it. The aim of the present study is to investigate alternatives to SCSA for the assessment of DNA damage induced by reactive oxygen species in bovine sperm. Five semen samples were incubated for 1 hour with hydrogen peroxide at concentrations ranging from 0 to 100 mM. DNA damage was assessed with SCSA, Sperm Chromatin Dispersion (SCD) and Sensitive Recognition of Individual DNA Ends (dSTRIDE), a recently-developed method for detecting DNA double strand breaks. We found that both SCSA and SCD detected a significant increase in DNA damage when spermatozoa were exposed to high concentrations of H_2O_2 ($\geq$ 10 mM), compared to the control ($p < 0.05$). dSTRIDE, however, did not detect an increase in DNA fragmentation under these conditions. SCD was therefore considered a possible alternative to SCSA.

Keywords

Bovine sperm; DNA fragmentation; oxidative stress; SCSA; SCD

Introduction

For the bovine livestock industry, whose activity depends on the insemination of many females with sperm obtained from a very limited number of males, the latter selection proves decisive to avoid suffering from a

substantial financial loss [1]. The use of sperm DNA fragmentation (SDF) as a parameter for semen quality assessment has seen increasing interest over the last decade from farm animal breeding companies, as well as fertility clinics, as high SDF has been shown to correlate with poor pregnancy outcomes [2]. However, while a negative correlation between the degree of DNA fragmentation and fertilization capacity has been demonstrated in humans, the real impact of sperm DNA integrity in farmed animals remains unclear, due to the paucity of studies on this topic [3]. Nevertheless, levels of SDF and conception rates were found to be anti-correlated in bulls [4–6], possibly because of the inability of the fertilized oocyte to repair excessively damaged sperm DNA [7]. As in humans [8], the reactive oxygen species (ROS) produced during freezing and thawing procedures are thought to be one of the major causes of SDF observed in cryopreserved semen from breeding bulls [9]. Therefore, for accurately measuring SDF in bull sperm, it is essential to use methods able to detect damages induced by ROS such as hydrogen peroxide (H_2O_2) as sperm H_2O_2 levels have been shown to negatively correlate with DNA integrity after cryopreservation [9].

Several tests have been developed for measuring the level of SDF in a population of treated or non-treated spermatozoa, and each of them presents advantages and disadvantages [10]. The sperm chromatin structure assay (SCSA®) is usually considered a gold standard for the assessment of DNA fragmentation in spermatozoa, as it has been extensively tested and validated in various species, including humans and farm animals [11,12]. SCSA is an indirect, flow cytometry-based method that relies on the incorporation of the fluorescent dye acridine orange (AO) in denatured sperm DNA [12,13]. The denaturation of DNA is achieved by acidic treatment and simultaneous heating of the sperm samples. To what extent the DNA is denatured in individual spermatozoa after treatment is believed to reflect the level of fragmentation of the molecule caused by single-strand breaks (SSBs) or double-strand breaks (DSBs), indistinctively [14,15]. A major advantage of the method is that it can investigate upwards of 10 000 spermatozoa in a few seconds [16]. However, it requires expensive equipment and highly qualified personnel, which can represent a significant barrier to its use [10]. In addition, a recent study showed that SCSA was unable to detect increases in DNA fragmentation in spermatozoa treated with high concentrations of hydrogen peroxide (H_2O_2), indicating that its use may not be suitable for all types of research questions related to SDF [17].

On the other hand, Sperm Chromatin Dispersion (SCD)is considered to be relatively simple and inexpensive, and is widely used in fertility clinics [18]. As for SCSA, it is an indirect method relying on the sensitivity of DNA to acidic denaturation, which increases with the level of both SSBs and DSBs [19]. In SCD, spermatozoa with intact DNA strands produce a characteristic halo of dispersed DNA loops after acid treatment and removal of nuclear proteins [20]. Spermatozoa with fragmented DNA will fail to produce such a halo, making it possible to determine their proportion within a population, using a simple microscope. Modified versions of SCD have been validated for use in several species [21–24]. However and to our knowledge, only a refinement of this technique that does not include an acidic denaturation step [25] has been tested and compared to SCSA

in bulls, using the Halomax© kit (Halotech DNA, Spain) [6,26]. Therefore, the original SCD method remains to be tested in cattle.

Interestingly, a fluorescence microscopy-based method abbreviated STRIDE (Sensitive Recognition of Individual DNA Ends) has been recently developed for counting the number of DNA breaks in fixed cells *in situ* [27]. Two different protocols have been developed for the assessment of SSBs (sSTRIDE) and DSBs (dSTRIDE). Both rely on the incorporation of labelled nucleotides at damage sites, followed by their detection using a proximity ligation assay (PLA) procedure[28], which will generate discrete and bright signals countable with a 3D fluorescent microscope. In dSTRIDE, the labelled nucleotides are incorporated using the terminal deoxynucleotidyl transferase (TdT) enzyme, similar to the TdT dUTP nick end labeling (TUNEL) assay. The authors showed in their study that dSTRIDE is specific for DSBs in somatic cells and allows their detection and counting on a single-cell level [27]. It has also been shown to work in human spermatozoa [27].

In the present study, the aim was to examine the use of SCD or dSTRIDE as alternative methods to SCSA, in measuring H_2O_2-induced DNA fragmentation in bull spermatozoa. Bull spermatozoa were treated with increasing amounts of H_2O_2, and the level of DNA fragmentation was measured using the three different methods.

Material and Methods

Sample collection

Bull semen samples from one Charolais and four Norwegian Red cryopreserved in commercial semen extender were obtained from Geno SA (Hamar, Norway). The Charolais sample was diluted with OptiXCell extender (IMV technologies, L'Aigle, France) whereas the Norwegian Red samples were diluted in Biladyl® (Minitube, Verona, WI, USA) and were frozen in classic bovine straws (IMV technologies, L'Aigle, France) with liquid nitrogen, following preliminary assessment of sperm concentration and motility. The bulls were part of regular semen production, and therefore, approval from an ethical committee was not necessary.

Sample preparation

Semen samples were thawed at 37 °C for 1 minute. A total of 84 million spermatozoa from each bull were added to 15 mL, polypropylene Falcon tubes containing 10 mL phosphate-buffered saline (PBS). Spermatozoa were separated from the semen extender by centrifugation at 400 × *g* for 10 minutes. The supernatant was transferred to a new tube and an additional centrifugation was performed to collect a larger portion of spermatozoa. The resulting cell pellets for each bull were resuspended in 2 mL PBS, combined, and distributed into four different tubes containing 4 mL PBS, for a total volume of 5 mL per tube. After centrifugation, the cell pellets were resuspended in 2 mL PBS alone (control) or containing 1 mM, 10 mM or 100 mM H_2O_2, and incubated for 1 hour at 37 °C. After treatment, the cells were washed twice in PBS and centrifuged as before.

The cell concentrations were measured using the Neubauer improved chamber (40442, Hecht Assistent, Sondheim vor der Rhön, Germany) and samples were aliquoted according to the required number of cells for each method. The aliquots were snap-frozen in liquid nitrogen and stored at -80 °C until further use.

Sperm Chromatin Structure Assay (SCSA)

The chromatin integrity of sperm samples was analyzed by SCSA® as described by Evenson and Jost (2001) [29] and Narud et al. (2022) [30] using a Cell lab Quanta TM SC MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with a 22 mW argon laser with excitation at 488 nm. Flow-checkTM beads (6605359, Beckman Coulter) were run daily. The method was performed in duplicate for each sample.

Thawed aliquots were diluted to 2×10^6 sperm/mL in TNE buffer (10 mM tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4) in a final volume of 200 µL. Thereafter, the sample was mixed with 400 µL acid detergent solution (0.38 M NaCl, 80 mM HCl, 0.1 % (w/v) Triton X-100, pH 1.2). The samples were incubated for 30 seconds at room temperature, followed by adding 1.2 mL AO staining solution (6 µg/ml) (A3568, Life Technologies, OR, USA) in a buffer containing 37 mM citric acid, 0.126 M Na_2HPO_4, 1.1 µM EDTA and 0.15 M NaCl, pH 6). At the end of 3 minutes set up mode, data acquisition was started, in which 5000 events were collected for each sample at a flow rate of ~ 200 events/second. Signals were separated by a 550-nm dichronic long pass mirror before being collected through a 525-nm bandpass filter (green), and a 670-nm long pass filter (red). The flow cytometry instrument was AO saturated by running AO equilibration solution (1.2 mL AO staining solution and 400 µL acid detergent solution) through the system for at least 5 minutes, prior to sample analysis. A reference semen sample from a bull of known DNA fragmentation index (DFI) was used to control the stability of the laser by setting the mean green and red fluorescence signals to 425 nm ± 5 and 125 nm ± 5, respectively, in a bivariate cytogram. This was performed both at the start of the analysis and after every fifth sample analyzed. On a linear scale, FL1 (green) was presented on the x-axis and FL3 (red) on the y-axis of the cytogram. By using FCS Express 6 Flow cytometry Software (Denovo Software, Los Angeles, CA, USA) the percentage of red (ssDNA) and green (dsDNA) fluorescence was determined. Based on a histogram of the fluorescence ratio red / (red + green), the DNA fragmentation index (DFI, %) was calculated. High DNA stainability (HDS, %) which corresponds to the spermatozoa with the most intensive green fluorescence recognized as immature spermatozoa, was determined by using the bivariate cytogram [15].

Sperm Chromatin Dispersion (SCD)

The SCD test was performed using the GoldCyto Sperm DNA kit (GoldCyto Biotech, GuangZhou, China) according to the manufacturer's protocol. The aliquots were thawed at room temperature and 30 mL of the cell suspension was mixed with a low-melting-point agarose, added onto slides pre-treated with agarose, and placed at 4°C for 5 minutes to solidify. The slides were then immersed in acid denaturation solution for 7 minutes at room temperature, in the dark. The solution contains hydrochloric acid to generate restricted single-

stranded DNA motifs from DNA breaks. Following the denaturation, the slides were transferred and immersed in a lysis solution for 25 minutes at room temperature to remove the nuclear proteins. The lysis was then stopped by placing the slides in distilled water for 5 minutes and dehydrated by immersion in sequential 70%, 90%, and 100% ethanol baths for 2 minutes each. The slides were air-dried before staining in 0.8 mL TA solution for 1 minute and 2.4 mL TB solution for 6 minutes. After staining, the slides were rinsed in tap water, air-dried and mounted with Eukitt (#253681, Panreac, Barcelona, Spain). Examinations of the slides were performed under a bright light microscope (Olympus, Tokyo, Japan) at ×1000 magnification and a minimum of 200 spermatozoa were counted. Nuclei with large or medium halos were defined as non-fragmented, while those with small or no halo and degraded spermatozoa were defined as fragmented. The results were expressed as % DNA fragmentation.

Sensitive Recognition of Individual DNA Ends for DNA double breaks (dSTRIDE)

Samples were thawed at room temperature and volumes estimated to contain 2.5×10^5 spermatozoa were applied to coverslips pre-coated with poly-L-lysine (Sigma-Aldrich, Saint-Louis, USA). Dried coverslips were placed in 24-well plates (Thermo Fisher, Waltham, USA) containing 0.5% bovine serum albumin (BSA) in PBS for 5 minutes on ice. The coverslips were immersed in 1 mL of 0.5% paraformaldehyde in PBS to stabilize the chromatin and kept on ice in the dark for 10 minutes. Two 5-minute washes with 0.5% BSA in PBS were performed on ice, before the coverslips were immersed in 200 µL decondensation solution (0.1% (w/v) Triton X-100, 5 mM dithiothreitol (DTT) in PBS) and kept at 25 °C for 15 minutes in the dark. Coverslips were rinsed with ice-cold PBS for 2 minutes, immersed in ice cold 70% ethanol, and fixated at -20 °C for 45-60 minutes.

Washes prior to the blocking step were performed using the wash and rinse buffers from the APO-BrdU™ TUNEL assay kit (Invitrogen, A23210), whereas the latter washes were performed using wash buffers from the Duolink® In Situ Wash buffers (Sigma-Aldrich, DUO82049). After fixation, one wash was performed with the wash buffer supplied from the kit, before BrdU was incorporated at double strand break (DSB) sites by the enzyme terminal deoxynucleotidyl transferase (TdT) at 37 °C for 1 hour. The remnants of the BrdU solution were rinsed away twice, with rinse buffer supplied with the kit. The coverslips were placed in 1 mL of blocking solution containing 2% BSA and 0.01% Tween 20 in PBS, overnight at 4 °C. Subsequently, the coverslips were placed upside down on top of 35 µL primary antibody solution containing anti-BrdU antibodies (#GR30168532, #GR33086028, Abcam, Cambridge, UK) raised in rabbit (1:200) and mouse (1:500), diluted in blocking solution. The incubation lasted for 45 minutes at room temperature, followed by three 5-minute washes. The proximity ligation assay including incorporation of PLA probes, ligation and signal amplification was performed according to the manufacturer's protocol [31]. Distilled water was utilized instead of the antibody diluent during the incorporation of the PLA probes. Following amplification, coverslips were immersed in 4'6-diamidino-2-phenylindole (DAPI) (50 mg/mL) diluted in wash buffer B for 5 minutes at

room temperature. Finally, coverslips were rinsed with the aforementioned buffer prior to mounting on glass slides using Mowiol 4-88 (Sigma-Aldrich, Saint-Louis, USA).

Image acquisition for dSTRIDE analysis

Images for quantifying the dSTRIDE signals were acquired using a 60x oil immersion objective mounted on a Leica SP8 TCS confocal microscope, driven by the Leica Application Suite software (LAS X, Danaher, USA). For each technical replicate, 10 fields containing at least 20 isolated nuclei were randomly selected based on DAPI staining. For each field, two parallel stacks of 16-bits, 1024x1024 pixels images were acquired along the z-axis, with a 0.30 µm z-step, one for collecting the DAPI signal and the other one for the dSTRIDE signals. The number of images was the same for all stacks of the same experiment and was established after finding the top and bottom of the nuclei present in the first acquired field, using the DAPI signal. DAPI and dSTRIDE signals were collected using a 405 nm and a 515 nm laser, respectively. For the 405 nm laser, the power was set to 1%, and for the 515 nm laser to 0.3 %, for all experiments. The gain was adjusted for each experiment to obtain the best signal over noise ratio and was kept the same for all acquisitions across the same experiment. All images were exported from LAS X as TIF files and further analyzed using the Image J software with a self-developed macro to automate the quantification process. Selection of appropriate nuclei was facilitated by the macro and approved by a single operator. Overlapping nuclei and nuclei on the boarder of the images were excluded prior to quantification. A minimum of 200 spermatozoa were analyzed per experiment.

Statistcal analyses

Statistical analyses were performed using SPSS analytical software version 27.0 (IBM Corp, Armonk, NY, USA). Data were not tested for normality as the data sets were too small ($n < 16$). As such, data were presented as median and quartiles 1 (Q1) and 3 (Q3). Wilcoxon Signed-Rank tests were conducted for pairwise comparisons of the parameter medians. *P*-values were adjusted for multiple comparisons using the Bonferroni method. A p-value < 0.05 was considered statistically significant.

Results

Sperm DNA integrity parameters assessed by SCSA after H_2O_2 incubation

For testing the ability of SCSA, SCD and dSTRIDE to measure different degrees of DNA fragmentation in bovine sperm, we exposed spermatozoa from 5 different bulls to increasing amount of H_2O_2, with concentrations ranging from 1 mM to 100 mM. For each condition, the level of sperm DNA fragmentation was assessed using the three methods in parallel and compared to a control of non-exposed spermatozoa from the same bull. Using SCSA, we first observed that all samples that were incubated without H_20_2 harbored a low percentage of spermatozoa with high DNA fragmentation index (% DFI), ranging from 1.56 to 2.31 % DFI (Figure 1A-

B; Table 1). Next, we found that exposing bovine semen samples to H_2O_2 concentrations over 1 mM increased the % DFI, compared to the control group (Figure 1, $p < 0.05$). In addition, the level of DNA damage measured by SCSA increased in response to increasing amounts of H_2O_2, in particular between 1 mM and 10 mM. However, even though the difference was found to be statistically significant, we only measured an overall 1.3-fold increase in % DFI in cells treated with 1 mM H_2O_2 compared to non-treated spermatozoa. Increasing H_2O_2 concentrations also influenced other SCSA parameters, including mean DFI, whose value increased in the aliquots treated with increasing concentrations of H_2O_2, and SD DFI which increased for the 100 mM H_2O_2 aliquot compared to the other values. Median values for each sperm parameter after H_2O_2 exposure are presented in Table 1.

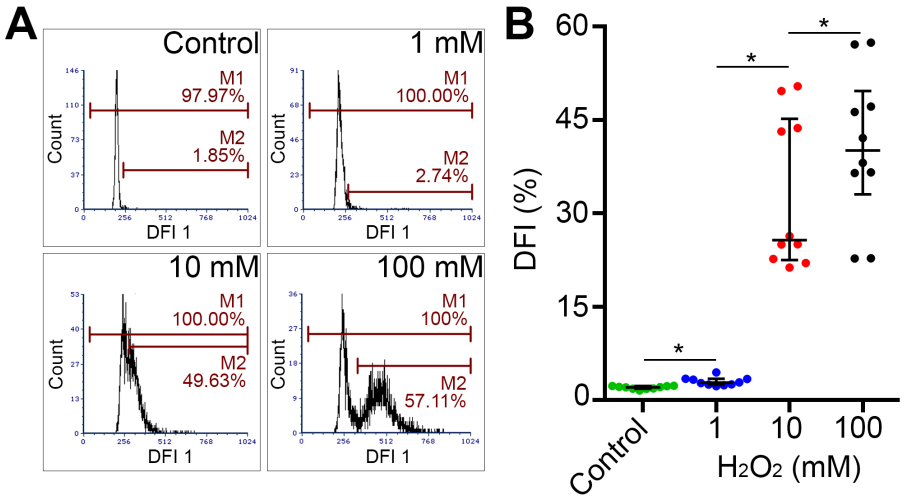


Figure 1. Increase in sperm chromatin damage in bovine semen samples after exposure to different concentrations of hydrogen peroxide (H_2O_2) assessed by SCSA. The experiment was repeated with semen samples from five bulls in total, in duplicate for each. (**A**) Scatterplot acquired by the FCS Express 6 Flow cytometry Software after SCSA. Marker M1 represents the total amount of cells localized in the gated region and M2 represents the population of cells with moderate or high DNA fragmentation index (% DFI). (**B**) DFI in percent after different H_2O_2 exposure groups. Median, quartile 1 (Q1) and quartile 3 (Q3) as lower and upper whiskers are indicated. * Significant difference, $p < 0.05$ obtained by Wilcoxon signed-rank test.

Table 1. Sperm chromatin integrity assessed by Sperm Chromatin Structure Assay (SCSA) after 1 hour incubation at 37 °C with different concentrations of H_2O_2 and without H_2O_2 as control. Values are presented as median and quartile 1 (Q1) and 3 (Q3) of five bovine semen samples, assessed in duplicates.

	Median (Q1 – Q3)			
Sperm parameter	**Control**	**1 mM**	**10 mM**	**100 mM**
Mean DFI	211.2 (207.3-213.9)[a]	221.4 (220.7-223.1)[b]	258.0 (251.4-280.9)[c]	279.2 (269.6-318.2)[d]
% DFI	2.1 (1.8-2.3)[a]	2.8 (2.4-3.4)[b]	25.7 (22.5-45.2)[c]	40.1 (36.5-46.9)[d]
% HDS	0.9 (0.7-1.3)[a]	0.9 (0.6-1.0)[a]	0.7 (0.5-0.9)[a]	0.7 (0.6-1.1)[a]
SD DFI	27.8 (25.3-32.0)[a]	29.4 (27.0-31.0)[a]	34.0 (31.0-44.7)[a]	39.0 (34.4-75.6)[b]

Abbreviations: Mean DFI: mean value of DNA fragmentation index; % DFI: % of sperm with moderate or high DNA fragmentation index; % HDS: % of sperm with high DNA stainability; SD DFI: standard deviation of DFI. [a-d] Values in rows with different letters differ significantly ($p < 0.05$), as assessed by Wilcoxon signed-rank test.

Increased amount of sperm DNA fragmentation after H_2O_2 exposure assessed by SCD

When using SCD we first confirmed that the untreated semen samples of all five bulls harbored a low proportion of spermatozoa with fragmented DNA (% DNA fragmentation), ranging from 3.3 ± 2.3 % to 6.7 ± 2.5 % (Figure 2A-B; Table 2).We observed an increase in % DNA fragmentation in all the aliquots exposed to H_2O_2 compared to the control group (Figure 2; Table 2). As for SCSA, the degree of DNA fragmentation measured for SCD was dependent on the concentration of H_2O_2, with a pronounced increase between the 1 mM and 10 mM conditions (Figure 2B). However, in contrast to SCSA, SCD was able to detect a 4-fold increase (comparison of the medians) in the proportion of spermatozoa harboring DNA fragmentation in the aliquot treated with 1 mM H_2O_2 compared to the control. When comparing the % DNA fragmentation assessed by SCD with the % DFI assessed by SCSA, the median value of % DNA fragmentation in the aliquots exposed to 10 mM H_2O_2 was approximately 3.5 times higher by SCD, compared to the assessment by SCSA, and approximately 2.2 times higher in the aliquots exposed to 100 mM H_2O_2 (Figure 2C).

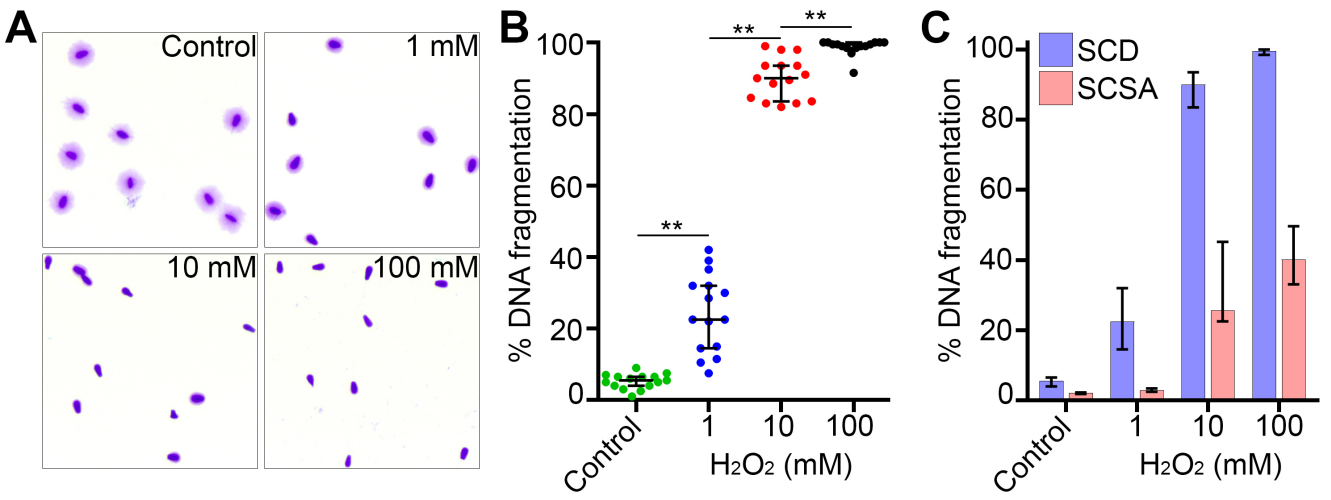


Figure 2. Increase in DNA fragmentation in bovine semen samples after exposure to different concentrations of hydrogen peroxide (H_2O_2), as assessed by sperm chromatin dispersion (SCD). The experiment was repeated with semen samples from five bulls in triplicates. (**A**) Characteristic halos present in spermatozoa with intact DNA after acid denaturation and the removal of nuclear proteins, visualized by bright field microscopy. (**B**) % of spermatozoa with a no observable halo, in different H_2O_2 exposure groups. Median, quartile 1 (Q1) and quartile 3 (Q3) as lower and upper whiskers are indicated. ** Significant difference, $p < 0.01$ obtained by Wilcoxon signed-rank test. (C) Comparison of % DNA fragmentation in sperm assessed by SCD and SCSA after exposure to different concentrations of hydrogen peroxide (H_2O_2). The values are represented as median, and the whiskers represent the lower (Q1) and upper (Q3) quartiles. The experiments were repeated with semen samples from five bulls, in triplicates for SCD and duplicates for SCSA.

Table 2. Sperm chromatin integrity assessed by Sperm Chromatin Dispersion (SCD) after 1 hour incubation at 37 °C with different concentrations of H_2O_2 and without H_2O_2 as control. Values are presented as median and quartile 1 (Q1) and 3 (Q3) of five bovine semen samples, assessed in triplicates.

	Median (Q1 – Q3)			
Sperm parameter	**Control**	**1 mM**	**10 mM**	**100 mM**
% DNA fragmentation	5.5 (4.0-6.5)[a]	22.5 (14.5-32.0)[b]	90.0 (83.5-93.5)[c]	99.5 (98.5-100.0)[d]

[a-d] Values in rows with different letters differ significantly ($p < 0.01$), as assessed by Wilcoxon signed-rank test.

No increase in DNA fragmentation after H_2O_2 exposure identified by dSTRIDE

In the last series of experiments, treated semen samples were submitted to the dSTRIDE method for visualizing, *in situ*, the occurrence of DNA DSBs. As expected, dSTRIDE generated discrete and bright fluorescent signals in the nucleus of a subpopulation of spermatozoa in each sample, with the number varying from one to more than 15 (Figure 3A). Consistent with our SCD and SCSA data, we detected DSBs using dSTRIDE only in a small fraction of the untreated spermatozoa, ranging from 3.6 ± 0.9 % to 9.0 ± 2.1 % (Figure 3B; Table 3). However, in contrast to the results obtained with the two previous methods, exposure to H_2O_2 did not increase the percentage of dSTRIDE-positive cells in bovine spermatozoa, independently of H_2O_2 concentration (Figure 3B). Median values for the % of spermatozoa with more than one dSTRIDE foci are presented in Table 3.

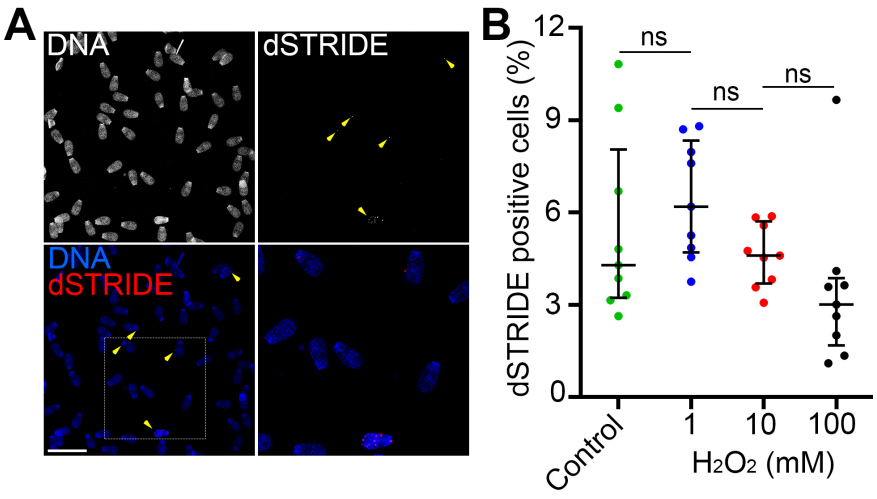


Figure 3. No significant changes in the % of dSTRIDE-positive cells in bovine semen samples after exposure to different concentrations of hydrogen peroxide (H_2O_2). The experiment was repeated with semen samples from five bulls in total, with the results of three bulls in triplicates included in the figure. (**A**) dSTRIDE foci in spermatozoa, visualized by 2D epifluorescence microscopy. Nuclear DNA was counterstained with DAPI. Scale bar, 20 µm. (**B**) % of spermatozoa with a positive dSTRIDE result in different H_2O_2 exposure groups. Median, quartile 1 (Q1) and quartile 3 (Q3) as lower and upper whiskers are indicated. Statistical significance was assessed using Wilcoxon signed-rank test. ns, not significant.

Table 3. Double-strand breaks assessed by Sensitive Recognition of Individual DNA Ends (STRIDE) in sperm after 1 hour incubation at 37 °C with different concentrations of H_2O_2,. and without H_2O_2 as control. Values are presented as median and quartile 1 (Q1) and 3 (Q3) of three bovine semen samples, assessed in triplicates.

	Median (Q1 – Q3)			
Sperm parameter	**Control**	**1 mM**	**10 mM**	**100 mM**
% dSTRIDE positive cells	4.3 (3.2-8.1)[a]	6.2 (4.7-8.4)[a]	4.6 (3.7-5.7)[a]	3.0 (1.7-3.9)[a]

[a] Values in rows with different letters differ significantly ($p < 0.05$), as assessed by Wilcoxon signed-rank test.

Discussion

The aim of this study was to evaluate if SCD and dSTRIDE could be used as alternative methods to SCSA for the assessment of DNA fragmentation in bovine semen samples after exposure to different concentrations of the oxidative agent H_2O_2. We observed a significant difference between all groups when the aliquots were assessed by SCSA and SCD, but not with dSTRIDE. Although both methods showed similar trends in their

dose-response relationship to H_2O_2, the median values corresponding to the percentage of spermatozoa with fragmented DNA was found to be between 2 and 8-fold higher with SCD compared to SCSA. We hypothesize that this difference may be attributed to the combination of nuclear proteins removal and acidic denaturation of DNA in the SCD protocol, which would allow a greater consideration of DNA breaks in highly condensed parts of the chromatin, otherwise inaccessible.

To our knowledge, only a single investigation has previously been published that compares the SCSA and SCD methods in bull semen [26]. In this study, the two methods were compared using semen from fighting bulls, incubated in $FeSO_4$ and sodium ascorbate for 6 hours to induce DNA fragmentation, The authors found that SCD had a higher repeatability coefficient (5.9) than SCSA (1.8), thereby suggesting a lower repeatability for SCD. Consistent with our results, although they used a different SCD kit than in the present study, they observed a lower spread in the data for SCSA compared to SCD, suggesting that SCSA is more precise in its measurements. Noteworthy, only one article has been published using the same GoldCyto sperm kit as here for SCD measurements, as of December 2023 [32]. However, the kit was used on human semen samples and did not discuss its validity. Therefore, our study is the first one to test the use of the GoldCyto sperm kit for the assessment of sperm DNA fragmentation, in bulls in particular.

The observed increase in % DFI in samples with increasing concentrations of H_2O_2 assessed by SCSA differs from the results of a recent work with a similar study design [17]. Indeed, while an approximately 1.2-fold increase in mean % DFI was measured in the semen sample exposed to 50 µM H_2O_2 compared to the control, Bittner-Schwerda et al. observed stagnation in % DFI in subsequent samples exposed to higher H_2O_2 concentrations (0.1 mM and 1 mM). We observed a similar increase in the median % DFI, with a 1.3-fold increase from the incubated control to our lowest exposed group (1 mM). However, in contrast to Bittner-Schwerda et al., we observed a pronounced increase in median % DFI in our highest H_2O_2 condition (100 mM) compared to the incubated control, with a 21-fold increase. The differences between studies could be explained, at least partially, by the use of different ranges of H_2O_2 concentrations. Another possibility based on the results from Bittner-Schwerda et al. is that exposing spermatozoa to H_2O_2 induces increased compaction of their chromatin, making it more difficult to open by acidic denaturation and, therefore, preventing the detection of a fraction of DNA breaks by SCSA [17]. In that case, the level of DNA fragmentation induced by exposure to H_2O_2 would most likely be underestimated when assessed by SCSA, in particular at low H_2O_2 concentrations. Finally, we cannot exclude the possibility that the difference in baseline % DFI between the two studies may also be attributed to the use of different bull breeds.

Our study includes methods that measure DNA fragmentation based on different underlying principles. SCSA and SCD are considered indirect methods because they rely on the consequences of DNA breaks on the structure of the DNA molecule [33]. Indeed, both methods measure how susceptible a cell's DNA is to acid

denaturation, which is |affected by the occurrence of SSBs and DSBs, indiscriminately [12,34]. On the other hand, dSTRIDE can be considered a direct method as the enzyme incorporates nucleotide analogues at DSB sites, which can be visualized and counted using a fluorescent microscope [27]. It is not known how much DSBs contribute to the % DFI compared to SSBs, nor is it known how many DNA breaks a cell would need to be considered fragmented by methods such as SCD and SCSA. And yet, knowing how many DNA breaks are present in a nucleus, as well as the proportion of SSBs and DSBs, could be of value, as DSBs present in the paternal genome have greater consequences for potential offspring than SSBs [2]. This is why we investigated dSTRIDE as another alternative method to SCSA, as it was developed to selectively detect DSBs [27].

H_2O_2 is thought to be 2000-fold more efficient at producing SSBs than DSBs, which means that few DSBs are produced even though large numbers of SSBs are present in the DNA [35]. In that aspect, our dSTRIDE data supports the developers' claim that dSTRIDE measures DSBs specifically, as a drastic increase in DSBs is not to be expected in spermatozoa exposed to H_2O_2. However, Kordon et al. observed a significant increase of DSBs in somatic cells when exposed to 4 mM H_2O_2 for 30 min [27], which is in the range of what was used in our study. The high compaction of chromatin in spermatozoa compared to somatic cells [36], accentuated by the exposure to H_2O_2 [17], may prevent large molecules such as enzymes or antibodies from accessing most DSB sites. This could explain why we did not detect any increase in the proportion of spermatozoa harboring positive dSTRIDE signals after exposure to H_2O_2 compared to the control group. The original dSTRIDE protocol would, in that case, not be suitable in spermatozoa to assess H_2O_2-associated DNA damage. For future investigation and validation for dSTRIDE as an additional method for the quantitative assessment of sperm DNA fragmentation, it would be of interest to expose spermatozoa to increasing amounts of ionizing radiation as it has been proven to directly induce double-strand breaks by breaking phosphodiester bonds within the DNA molecule [37]. It could also be interesting to develop a protocol dedicated to the use of dSTRIDE in spermatozoa. This would include an upstream step allowing the partial decondensation of chromatin to facilitate its in-depth access to the different proteins used in the method,

Conclusion

There is a need for reliable methods to evaluate DNA fragmentation in spermatozoa, along with an understanding of their strengths and limitations. It is also important to be aware of the conditions under which the experiments were performed. The present study shows that SCD can be considered an alternative to SCSA for the assessment of DNA fragmentation in bovine samples. Both methods had an increasing response in level of DNA fragmentation to increasing concentrations of the DNA damaging agent H_2O_2, with a more sensitive response by SCD. dSTRIDE was not observed to be a good alternative to SCSA in the context of DNA fragmentation caused by H_2O_2, but future studies more directed towards the assessment of DSBs should be conducted to provide greater insight into the method.

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Author contributions

Conceptualization, E.D., O.W., and I.AA.; methodology, E.D.; software, I.AA.; formal analysis, I.AA.; investigation, I.AA., M.I., E.B.S., A.H.A.K.; resources, A.H.A.K. and I.AA.; writing—original draft preparation, I.AA. and M.I; writing—review and editing, E.D., O.W. and A.H.A.K.; visualization, E.D.; supervision, E.D. and O.W. All authors have read and agreed to the published version of the manuscript.

Data availability statement

Data are contained within the article. Raw data will be made available on request.

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Ethical considerations

Ethical review and approval were waived for this study as the bulls were part of regular semen production.

Conflicts of Interest

The authors declare no conflict of interest.