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Calcium and vitamin D homoeostasis in male fertility

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Calcium and vitamin D have well-established roles in maintaining calcium balance and bone health. Decades of research in human subjects and animals have revealed that calcium and vitamin D also have effects on many other organs including male reproductive organs. The presence of calcium-sensing receptor, vitamin D receptor, vitamin D activating and inactivating enzymes and calcium channels in the testes, male reproductive tract and human spermatozoa suggests that vitamin D and calcium may modify male reproductive function. Functional animal models have shown that vitamin D deficiency in male rodents leads to a decrease in successful mating and fewer pregnancies, often caused by impaired sperm motility and poor sperm morphology. Human studies have to a lesser extent validated these findings; however, newer studies suggest a positive effect of vitamin D supplementation on semen quality in cases with vitamin D deficiency, which highlights the need for initiatives to prevent vitamin D deficiency. Calcium channels in male reproductive organs and spermatozoa contribute to the regulation of sperm motility and capacitation, both essential for successful fertilisation, which supports a need to avoid calcium deficiency. Studies have demonstrated that vitamin D, as a regulator of calcium homoeostasis, influences calcium influx in the testis and spermatozoa. Emerging evidence suggests a potential link between vitamin D deficiency and male infertility, although further investigation is needed to establish a definitive causal relationship. Understanding the interplay between vitamin D, calcium and male reproductive health may open new avenues for improving fertility outcomes in men.

Key words: Vitamin D: Calcium: Fertility: Male reproduction

Abbreviations: 1, 25$(OH)_2D_3$, 1, 25-dihydroxyvitamin D3; 25OHD, 25-hydroxyvitamin D; CaSR, calcium-sensing receptor; CYP, cytochrome P450; PMCA, plasma-membrane calcium ATPase; PTH, parathyroid hormone; TRPV, transient receptor potential vanilloid; VDR, vitamin D receptor.

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Male reproduction is of increasing interest and concern worldwide due to the growing issues with reproductive health and low fertility rates[1]. According to the UN, the average global fertility rate stood at 2·3 births per woman in 2021, falling from about 5 births per woman in the mid-twentieth century[2]. Male infertility contributes to the problem in almost 50 % of infertile couples, with up to 30 % of all cases being attributed to a male factor as the sole cause[3–5]. Numerous causes can influence male reproductive function. These include genetic mutations, environmental aspects, lifestyle choices, medical conditions or medications[6–9]. In recent years, the potential impact of nutritional factors on male reproductive health has gained significant attention. Both vitamin D deficiency and low-ionised calcium have been linked with impaired semen quality[10]. While it can be challenging to directly attribute instances of male infertility to dietary or lifestyle changes, these factors can contribute to the problem. Modern dietary habits have seen a shift towards processed foods[11], which are often low in essential nutrients and can lead to nutrient deficiencies that may have a negative influence on fertility.

Vitamin D is a steroid hormone essential for various physiological processes in many different organs beyond its classical role in maintaining calcium homoeostasis and bone health[12]. In the past 20 years, there has been a great interest in exploring the beneficial effects of vitamin D on human health and in various organs during health and disease[13–15]. It has been suggested to be involved in several aspects of male reproductive function, such as sperm production, sperm motility and hormonal regulation[16–18]. Generally, vitamin D deficiency is defined as serum 25-hydroxyvitamin D (25OHD) levels below 25 or 30 nM/l and vitamin D insufficiency as levels below 50 nM/l. The active vitamin D metabolite, 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) (calcitriol), exerts its effects through the vitamin D receptor (VDR), which is present in various reproductive tissues including testes, epididymis and prostate[9]. The VDR, upon activation by $1,25(OH)_2D_3$, potentially regulates the expression of genes involved in steroidogenesis, spermatogenesis and sperm maturation[8].

Vitamin D regulates serum calcium concentrations by enhancing intestinal absorption, utilising bone storage, and increasing renal reabsorption[8]. These actions are involved in the tight regulation of calcium in the body which is essential for almost all physiological functions. Calcium is not only crucial for skeletal integrity but is also involved in intracellular signalling pathways, muscle contraction and hormonal regulation. In the male reproductive system, calcium is essential for sperm motility, sperm capacitation and the acrosome reaction, which are crucial steps for successful fertilisation[10]. Disturbances in calcium homoeostasis can affect sperm function and contribute to male infertility[10,19]. Therefore, adequate levels of both vitamin D and calcium have been proposed to be important for optimal male reproductive health.

Understanding the significance of vitamin D and calcium in male reproductive health has the potential to guide clinical practice, form interventions and improve reproductive outcomes for men. As such, this review summarises the current understanding of the effects of vitamin D and calcium on male reproductive function.

Male reproductive tract

Male reproductive organs ensure the production, storage and delivery of motile and competent spermatozoa and the synthesis and secretion of male sex steroids. The testes are responsible for the production of spermatozoa through a complicated process known as spermatogenesis, which occurs in the seminiferous tubules[8]. Spermatogenesis involves both mitosis and meiosis to complete the development of mature spermatozoa from spermatogonial stem cells[20]. Spermatogenesis relies on the interplay between various cell types, including germ cells, Sertoli cells, peritubular cells and Leydig cells. Within the seminiferous tubules, the specialised Sertoli cells, in conjunction with peritubular cells, create the microenvironment that allows germ cells to develop from diploid spermatogonia to haploid spermatids[21]. They provide physical support and nutrition, and regulate the milieu required for proper sperm development. Various genetic and endocrine factors tightly regulate the functions of Sertoli and peritubular cells, with follicle-stimulating hormone and testosterone being prominent regulators necessary for optimal spermatogenesis[22]. Leydig cells, located in the interstitial spaces between the seminiferous tubules, produce testosterone in response to luteinising hormone stimulation. This ensures a high intratesticular testosterone concentration that can be up to 100 times higher than in serum[23,24]. Testosterone has a significant impact on fetal testis development, the descent of the testes into the scrotum and the maturation of Sertoli cells during puberty. The maturation of Sertoli cells marks the initiation of spermatogenesis and coincides with the cessation of their proliferation during puberty. Mature Sertoli cells cannot proliferate, thus the quantity of Sertoli cells established during early life determines the capacity for sperm production in adulthood[21]. Consequently, the number of sperm present in a semen sample is, to a certain extent, determined during fetal and early life.

After successful spermatogenesis, the spermatozoa are immotile and undergo passive transportation to the epididymis through the efferent ducts. In the epididymis, the spermatozoa undergo final maturation and become motile. The epididymal fluid composition is tightly regulated and differs markedly as the spermatozoa transit through the caput, corpus and cauda of the epididymis. During ejaculation, spermatozoa meet secretions from the prostate and seminal vesicles that may also influence sperm function.

Vitamin D

Physiology and metabolism

The inactive form of vitamin D, cholecalciferol, is synthesised in the skin following the conversion of

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7-dehydrocholesterol by UV B radiation from the sun[25,26]. Cholecalciferol can also be absorbed from the diet or via supplementation. In detail, cholecalciferol must undergo two hydroxylations to form the active form of vitamin D, $1,25(OH)_2D_3$ that can bind and activate the VDR[27–29]. These steps involve the microsomal cytochrome P450 (CYP) enzymes. The first step is 25-hydroxylation by hepatic CYP2R1 which forms the prohormone 25OHD (calcidiol) followed by 1α-hydroxylation by renal CYP27B1 to form $1,25(OH)_2D_3$, whereas CYP24A1 inactivates 25OHD and $1,25(OH)_2D_3$[30]. Parathyroid hormone (PTH) is the main inducer of renal CYP27B1 expression and thus the main inducer of circulating $1,25(OH)_2D_3$ and a powerful mobiliser of calcium from the skeleton[31]. $1,25(OH)_2D_3$ binds and activates the VDR which forms a heterodimer with the retinoid X receptor. This complex recognises vitamin D response elements in the promoter region of target genes and regulates transcription[30]. The DNA-binding domain of the VDR mediates the transcriptional effects, but VDR also has an alternative ligand-binding pocket that mediates rapid non-genomic effects by regulating second messenger systems. In clinical practice, vitamin D status is determined by measuring serum levels of 25OHD and not $1,25(OH)_2D_3$, as 25OHD correlates with bone mass density, serum calcium level and PTH secretion[32,33]. However, cellular responsiveness to circulating vitamin D metabolites goes beyond VDR expression as the presence of activating and inactivating enzymes locally in the target organs influences the availability of substrates for VDR and thereby influences the effect of vitamin D on the target tissue[34,35].

Expression of vitamin D regulating enzymes and vitamin D receptor in the male reproductive system

Studies have consistently found mRNA and protein expression of the VDR and the vitamin D activating and inactivating enzymes in various cell types of the gonads and male reproductive tract[9]. In most studies, VDR is co-expressed with the activating enzyme, CYP27B1, and the deactivating enzyme, CYP24A1. This co-expression occurs throughout the reproductive tract, including in some Sertoli cells, most germ cells, spermatozoa, Leydig cells and the epithelial cells lining the tract[36–42]. The local presence and activity of the enzymes play a crucial role in regulating the concentration of $1,25(OH)_2D_3$ within cells and consequently activating the VDR.

In adult testes, the VDR and metabolising enzymes are primarily expressed in human germ cells, with a marked expression in spermatogonia, spermatocytes and spermatids in both human subjects and rodents[18,43]. Studies have also found VDR expression in both the nucleus and cytoplasm of primary cultures of immature Sertoli cells, in fetal Sertoli cells and in the immature mouse Sertoli cell line TM4 where $1,25(OH)_2D_3$ is known to mediate fast non-genomic effects[44–50]. The expression levels of CYP2R1, CYP27B1 and CYP24A1 in the testes indicate that the cells are equipped to respond to and regulate the effects of vitamin D metabolites[8,18]. Furthermore, the effects of cholecalciferol or calcitriol treatment on gonadal function may vary depending on the specific expression pattern of these enzymes. Cholecalciferol can be activated locally, potentially eliciting a response, whereas calcitriol is already active and immediately triggers responses or undergoes inactivation. This suggests that the local regulation of vitamin D is important for spermatogenesis and sperm function. The expression of VDR in germ cells suggests that calcitriol may play a role in sperm function[51]. Multiple studies have reported the presence of VDR in specific regions of mature human spermatozoa, including the post-acrosomal part of the head, the neck region and the midpiece[18,52]. Additionally, the vitamin D activating enzymes are also expressed in spermatozoa, and the inactivating enzyme CYP24A1 exhibits a distinct expression pattern at the annulus. Notably, the expression levels of VDR and CYP24A1 are higher in spermatozoa from healthy men compared with infertile men[53], which supports that vitamin D may be beneficial for sperm function.

VDR expression has also been demonstrated in Leydig cells, suggesting a potential direct impact of vitamin D on steroidogenesis and thereby also an indirect effect on spermatogenesis and male fertility. Recent studies have detected VDR at the protein level in fetal and adult Leydig cells in human subjects, roosters and mice[18,38,54,55]. Moreover, VDR, CYP27B1 and CYP24A1 are all expressed in the epididymis, prostate and seminal vesicles[18,37,38], which highlights a potential direct effect throughout the male reproductive system.

Animal models: vitamin D deficiency and male fertility

Numerous animal models have been utilised to investigate the relationship between vitamin D and male fertility (defined as successful conception). An overview of the models is shown in Table 1. The first association was found in a study exploring vitamin D deficiency in rats[56]. Vitamin D deficiency in male rats led to a 45 % decrease in successful mating, defined as the presence of sperm in the vaginal tract, compared with rats with sufficient vitamin D levels. Additionally, rats with vitamin D deficiency had 71 % fewer pregnancies compared with vitamin D-sufficient rats[56]. When the rats were supplemented with calcium, the fertility potential was restored, implying an indirect effect of hypovitaminosis D leading to hypocalcaemia and secondarily reduced fertility[57]. However, after reassessment of the data by adjusting for the mating period and male:female ratio, and by determining the time to pregnancy, it was evident that the rats still had lower pregnancy rates after insemination with sperm from normocalcemic vitamin D-deficient rats than normocalcemic vitamin D-replete rats[57]. This indicates that vitamin D itself is important for semen quality and that the impaired fertility caused by vitamin D deficiency cannot be fully reversed by correcting hypocalcaemia alone[9]. Newer animal studies on vitamin D deficiency have supported the negative effects of inadequate vitamin D levels on semen quality and

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Table 1. Six studies investigating the relationship between vitamin D deficiency and fertility in rodents

Subgroup	Fertility	Sperm count	Sperm motility	Sperm morphology	Reference
Naval Medical Research Institute (NMRI) mice*	↓	N.D.	↓	→	Shahreza *et al.*[62]
Sprague Dawley rats	N.D.	↓	↓	↓	Zamani *et al.*[61]
Sprague Dawley rats	→	N.D.	↓	N.D.	Merino *et al.*[64]
Institute of Cancer Research (ICR) mice	→	↓	N.D.	N.D.	Fu *et al.*[60]
Sprague Dawley rats	↓	N.D.	N.D.	N.D.	Uhland *et al.*[57]
Sprague Dawley rats	↓	N.D.	N.D.	N.D.	Kwiecinski *et al.*[56]

N.D., not determined.
Animal models made vitamin D-deficient either by diet, knockout of the vitamin D receptor or the 1α-hydroxylase (*Cyp27b1*).
*Phenotype reported for prolonged vitamin D-deficient mice; ↓, negatively associated; →, no association.

male fertility. In general, the low fertility rates in vitamin D-deficient male rodents seem to be caused by impaired sperm count, motility and occasionally poor sperm morphology[58,59].

Dietary vitamin D-deficient mice have also been shown to have lower testicular weight and fewer spermatozoa in the cauda epididymis compared with controls[60]. Another study revealed adverse impacts on semen quality in vitamin D-deficient rats when compared with control groups. These effects included reductions in germ cell numbers, sperm count, sperm morphology, motility and viability. Furthermore, a decrease in serum testosterone levels was also observed in vitamin D-deficient rats[61]. Duration and severity of the deficiency may be an important factor in reproductive function. Vitamin D deficiency through one spermatogenic cycle induced a significant increase in sperm DNA fragmentation but no significant effect on other semen parameters[62]. In contrast, prolonged vitamin D deficiency through two spermatogenic cycles resulted in a modest but significant decrease in sperm motility. Furthermore, different degrees of vitamin D deficiency have been induced in mice, and no effect was observed on sperm concentration and sperm viability, but sperm morphology was decreased in the severe deficiency group[63]. In another study, DNA fragmentation has also been shown to increase significantly in spermatozoa of vitamin D-deficient rats, which could explain the impaired fertility[64]. Combined, these studies indicate that vitamin D is involved in the regulation of male fertility in rodents, suggesting a potential link between vitamin D status and reproductive health.

Human studies

Many of the cross-sectional studies investigating the link between vitamin D status and testicular function have been reviewed and discussed before, and the link critically depends on the fraction of men with low vitamin D status in the study population[65]. Recent years have seen a surge in human intervention trials with vitamin D and male fertility outcomes. In this review, we have evaluated six randomised clinical intervention studies, with four of them being conducted after 2020. An overview of the trials is shown in Table 2. In the first randomised controlled trial from 2014, 86 infertile men with idiopathic oligoasthenozoospermia were included and randomised to receive either a 3 month supplementation of cholecalciferol (5 μg daily) and calcium (600 mg daily), or placebo[66]. This study found a significant increase in the number of progressive motile sperm and a higher pregnancy rate in the vitamin D *v.* placebo-treated men. These findings are supported by the most recent randomised clinical trial of 120 men conducted in 2022 on Indian men[67]. They found an increased sperm volume, progressive motility, total motility and sperm count in men treated with cholecalciferol (100 μg daily for 10 weeks) when compared with the placebo group. The study does not report the serum 25OHD levels before or after treatment, so it is not possible to determine whether the effect was found in vitamin D insufficient, deficient or sufficient men.

In 2018, the biggest study to date was conducted and contradicted these findings[17]. In the Copenhagen bone-gonadal study, 330 Danish infertile men with vitamin D insufficiency (defined as serum 25OHD <50 nM/l) were randomised to receive either a high single-dose cholecalciferol supplementation (7 500 μg) followed by daily supplementation of cholecalciferol (35 μg) and calcium (500 mg daily), or placebo. The study showed that supplementation did not affect semen parameters or live birth rates when comparing the groups. The spontaneous pregnancy rate tended to be higher in couples in which the men were treated with vitamin D and calcium compared with couples in which the men were in the placebo group, but the results were not statistically significant. However, subgroup analysis of oligospermic men did demonstrate an increased live birth rate in the treatment group (36 *v.* 18 %). One criticism of the study is the use of a high-loading dosage that could lead to high CYP24A1 activity locally in many organs and thus inactivate vitamin D activity in the gonads. The overall negative findings of the study are consistent with three other recent randomised clinical trials conducted on Iranian men[68–70]. One trial with results published in 2020, investigated the effects of vitamin D supplementation in sixty-two infertile men with impaired semen quality and vitamin D insufficiency (defined as serum 25OHD <50 nM/l). The men were randomised to receive either cholecalciferol (1 250 μg once weekly) for 8 weeks, followed by a maintenance dose (1 250 μg once) lasting the final 4 weeks, or placebo treatment. The same study design was used by another Iranian research group from 2021, conducted on forty-four men with asthenozoospermia[68]. Both studies

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Table 2. Interventional studies of vitamin D and semen quality in infertile men

Population	*N*, treatment	*N*, control	Age	BMI	25OHD status	Vitamin D_3 dose	Fertility	Sperm count	Sperm motility	Sperm morphology	Reference
Indian	60	60	34(SD 8)	N.D.	N.D.	100 µg daily for 10 weeks	N.D.	↑	↑	↑	Padmapriya *et al.*[67]
Iranian	20	20	33(SD 1)	24·5(SD 0·9)	<75 nM/l	1 250 µg weekly for 8 weeks, followed by 1 250 µg once for final 4 weeks	N.D.	↑	↑	↑	Gheflati *et al.*[68]
Iranian	43	43	35(SD 5)	28·4(SD 3·0)	<75 nM/l	100 µg daily for 12 weeks Maghsoumi-Norouzabad *et al.*[69]	↑	↑	↑	↑	
Iranian	30	32	35(SD 5)	25·7(SD 1·9)	<75 nM/l	1 250 µg weekly for 8 weeks, followed by 1 250 µg once for final 4 weeks	↑	↑	↑	↑	Amini *et al.*[70]
Danish	164	166	35(SD 7)	26·5(SD 4·2)	<50 nM/l	7 500 µg as initial dose, followed by 35 µg and 500 mg calcium daily for 150 days	↑	↑	↑	↑	Blomberg Jensen *et al.*[17]
Chinese	43	43	N.D.	N.D.	N.D.	5 µg and 600 mg calcium daily for 12 weeks	↑	↑	↑	N.D.	Deng *et al.*[66]

25OHD, 25-hydroxyvitamin D; *N*, cohort size; N.D., not determined.
Six human randomised controlled studies investigating the relationship between serum vitamin D levels and semen quality in human subjects were evaluated.
↓, decreased with supplementation; ↑, increased with supplementation ; →, no changes.

used vitamin D-deficient men (defined as serum levels <75 nM/l) and found no effect of cholecalciferol supplementation on semen parameters. A third Iranian study published in 2021 evaluated the effects of cholecalciferol on both endocrine markers and semen parameters in eighty-six infertile men with asthenozoospermia[69]. Supplementation with 100 µg cholecalciferol daily for 12 weeks had no significant effects on semen volume, sperm count or sperm morphology compared with the placebo group. However, they found a positive effect on total and progressive sperm motility, to some extent supporting the results found in the Chinese and Indian study populations.

Overall, most of the interventional human data available indicate that vitamin D supplementation does not affect sperm count, volume, morphology or pregnancies. However, it must be noted that there are some contradictory reports on the presumed effect on total and progressive sperm motility, but positive data have only been generated in a few relatively small studies. Furthermore, with the diverse study populations of the mentioned studies (co-morbidities, age and BMI), design, inclusion criteria and baseline vitamin D status, it is hard to find consensus on the effect. Observational human studies and case–control studies investigating the association between vitamin D status and reproductive functions have also been conducted. These results are conflicting; however, most studies find that low vitamin D status is linked to impaired semen quality and fertility[20,71–76]. Therefore, it seems likely that the possible effect of vitamin D supplementation on semen quality and male fertility is present under conditions with vitamin D deficiency or prolonged vitamin D insufficiency. These data are in line with animal studies showing impaired fertility and poor semen quality in males with vitamin D deficiency, which by combining suggest that vitamin D deficiency should be avoided to secure optimal reproductive function.

Calcium

Systemic calcium homoeostasis

Serum calcium is maintained within a narrow physiological range to secure normal function of several organs as multiple cellular processes are influenced by calcium. Virtually all cells in the body have a 20 000-fold gradient of calcium ions (Ca^{2+}) from the extracellular to the intracellular environment (about 100 nM/l-Ca^{2+} intracellularly), making them capable of using Ca^{2+} as a second messenger when entry is permitted into the cell by Ca^{2+} channels[77]. The total calcium concentration in serum is 2·20–2·55 mM/l and the non-protein-bound ionised calcium level is 1·18–1·32 mM/l although the exact reference levels vary among laboratories. A large fraction of calcium is protein-bound or in complex with anions such as citrate, lactate, phosphate or bicarbonate and the free and active fraction is therefore dependent on pH and availability of binding partners[78]. To maintain serum calcium within this narrow range several potent regulators influence systemic calcium homoeostasis by

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modifying intestinal absorption, renal reabsorption and excretion and release from the skeletal reservoir. The calcium-sensing receptor (CaSR) in the parathyroid cells senses the Ca^{2+} concentration in serum and transduces an intracellular signal that inhibits the production and release of PTH into the bloodstream[79]. PTH binds primarily to the PTH receptor 1 and mobilises calcium to the circulation. In bone, PTH mediates calcium release by promoting bone resorption, and in the kidney, it increases calcium reabsorption and up-regulates CYP27B1 activity which ensures high vitamin D activity. In mice, the CaSR is indispensable as *Casr* knockout mice die shortly after birth and suffer from hypercalcaemia and hyperparathyroidism[80]. In human subjects, homozygous loss-of-function mutations in *CASR* cause neonatal severe hyperparathyroidism, a potentially life-threatening condition with severe hypercalcaemia, bone demineralisation and respiratory distress, whereas a heterozygous loss-of-function mutation in *CASR* causes familial hypocalciuric hypercalcaemia 1[81], which shows that full compensation by other factors does not occur.

In the epithelia lining the intestine and kidney, calcium is transported from the lumen to the blood through the cell cytoplasm by the transcellular pathway or between intercellular spaces by the paracellular pathway. Expression of transcellular calcium transporters in the kidney and intestine is regulated mainly by 1,25 $(OH)_2D_3$[82]. Transcellular calcium transport involves three steps: first, Ca^{2+} is transported into the cytosol by transient receptor potential vanilloid −5 or −6 (TRPV −5/−6) Ca^{2+} channels. Secondly, Ca^{2+} binds intracellularly to calbindin-D_{9k} (or -D_{28k}) to maintain a low intracellular Ca^{2+} concentration. Thirdly, Ca^{2+} is removed from the cytosol by the plasma-membrane calcium ATPase (PMCA) proteins or the Na^+/Ca^{2+} exchanger 1[83]. CaSR is expressed in the thick ascending limb of Henle in the kidney, and acute inhibition of CaSR increases the permeability of the paracellular Ca^{2+} pathway[84]. More recently, it has been shown that CaSR inhibits the paracellular Ca^{2+} transport by increasing the expression of renal claudin-14 responsible for the inhibition of reabsorption in a PTH-independent manner[85]. In addition, PTH directly increases renal Ca^{2+} reabsorption by inhibiting claudin-14[86].

Calcium balance in the male reproductive tract

Calcium and phosphate concentrations are very different in the proximal and the distal parts of the epididymis. The concentration of calcium decreases, whereas the concentration of phosphate increases[87]. These variations in calcium and phosphate levels are believed to play a crucial role in sperm maturation and the initiation of sperm motility and in keeping the sperm quiescent during storage in the distal epididymis. The high calcium concentration in the seminal fluid may be of great importance as spermatozoa are transcriptionally silent and heavily rely on intracellular calcium as a signalling system[88]. A list of observational studies exploring total and ionised calcium levels in the human seminal fluid/plasma is summarised in Table 3. Based on the weighted average of the studies included in Table 3, the concentration of total calcium in the seminal fluid is about 7·48 mM/l, and the concentration of ionised calcium is about 0·23 mM/l. Citrate and phosphate concentrations are also high in the seminal fluid, ensuring competent buffer systems and making the ionised calcium concentration lower than the corresponding serum level[89–93]. Studies exploring the potential impact of the total calcium concentration in seminal fluid on sperm parameters are inconclusive – some suggest it is associated with increased motility[94,95], another study found an inverse relationship with sperm morphology[96] and other studies found no impact on sperm function[97–99]. One study showed that low total calcium content in the seminal fluid (<5 mM/l) was associated with fewer progressive and total motile sperm and fewer morphologically normal sperm compared with men with calcium levels between 5 and 10 mM/l[19]. Regarding studies that investigated the link between ionised calcium and semen quality variables, two studies found a positive association with motility[95,97], one showed a negative association[100] and one study found no effect[91]. The possible link between total calcium/Ca^{2+} levels and sperm motility/morphology/concentration is interesting because these semen variables are predictors of male fertility potential. However, the spermatozoa are only briefly exposed to the seminal fluid before it is mixed with female fluids, which questions a direct effect of calcium content on the fertility potential. The total calcium concentration in seminal fluid is more than 2-fold higher than the level found in serum, and the levels are not associated (Fig. 1A)[19,100], hence there must be an active transport of calcium from serum into the seminal fluid. The calcium concentration in the different epididymal compartments (studies have only been conducted in rodents) varies largely from low to several-fold higher than the corresponding serum concentration (Fig. 1B)[101–103]. To maintain this steep gradient the mechanism and proteins underlying calcium homoeostasis must be tightly regulated. Seminal fluid comprises fluid from the epididymis (about 10 %), prostate (about 20 %) and seminal vesicles (about 70 %), which indicates that the calcium content in the ejaculate is highly dependent on secretions occurring after the transit and storage of the spermatozoa in the epididymis. At the time of ejaculation, spermatozoa escape the millimolar concentrations of citrate and phosphate in seminal fluid and encounter about 2·2 mM/l-total calcium and about 1·23 mM/l-Ca^{2+} in the follicular fluid in the female reproductive tract[91,104]. This influences sperm function and facilitates sperm capacitation and hyperactivation that help the sperm swim up in the vicinity of the oocyte.

Gene expression is silenced during spermatogenesis as histones are replaced by protamines that further condense DNA and prevent transcription within the small spermatozoa (about 4·4 × 2·8 μM)[105,106]. Protein synthesis and processing are also hampered and therefore, spermatozoa critically depend on second messenger systems to transmit extracellular signals, and they mainly rely on changes in the intracellular calcium concentration

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Table 3. Studies investigating ionised and total calcium levels in human seminal fluid/plasma

Seminal plasma/fluid (mM/l)				
Ionised calcium	Total calcium	Subgroup	Conclusions	Reference
0·19(SD 0·03)	5·13(SD 2·50)	Healthy young men (*n* 9)	CaSR is important for sensing Ca^{2+} in spermatozoa	Boisen *et al.*[104]
		Men recruited from a fertility clinic	Men with the lowest sperm motility had significantly lower Ca^{2+} levels in seminal fluid	Kılıç *et al.*[97]
0·16(SD 0·04)	5·60(SD 1·43)	Sperm motility <60% (*n* 45)		
0·37(SD 0·28)	5·48(SD 1·37)	Sperm motility >60% (*n* 30)		
0·18(SD 0·01)	3·30(SD 0·12)	Men referred to a fertility clinic	Men with the lowest sperm motility had significantly lower Ca^{2+} and total calcium levels in seminal fluid	Prien *et al.*[95]
0·23(SD 0·01)	3·42(SD 0·13)	Sperm motility <60% (*n* 15)		
		Sperm motility >60% (*n* 21)		
0·25(SD 0·07)		Men referred to an andrology unit for different reasons (*n* 27)	No association between ionised calcium concentrations and motility	Magnus *et al.*[91]
0·24(SD 0·02)	11·07(SD 0·41)	Healthy men (*n* 45)	N.A.	Ford and Harrison[90]
0·17(SD 0·05)	5·70(SD 2·50)	Men referred to a reproductive unit for different andrological reasons (*n* 32–37)	Spermatozoa from semen samples with Ca^{2+} levels below average had higher motility than spermatozoa in semen samples with higher levels. Ca^{2+} was not correlated with total calcium	Arver *et al.*[100]
	7·8(SD 3·90)	Subfertile men (*n* 165)	Men with a seminal fluid calcium concentration between 5 and 10 mM had higher sperm motility compared with men with a calcium concentration between 1 and 5 mM	Boisen *et al.*[19]
	6·79(SD 0·43)	Healthy men (*n* 24)	N.A.	Valsa *et al.*[133]
	4·86(SD 1·93)	Normozoospermic (*n* 30)	No difference in the calcium concentrations between normospermic and azoospermic semen samples	N'Guessan *et al.*[93]
	5·32(SD 1·23)	Azoospermic (*n* 30)		
	9·61(SD 3·76)	Healthy men, 18–55 years old (*n* 515)	Calcium was positively associated with sperm concentration	Liang *et al.*[134]
		Men referred to a fertility clinic	In the neurologically intact group, seminal calcium was negatively correlated with sperm morphology	Salsabili *et al.*[96]
	4·55(SD 4·28)	Spinal cord-injured (*n* 93)		
	5·39(SD 4·94)	Neurologically intact (*n* 145)		
	11·88	Healthy men (*n* 50)	Low calcium was associated with better CASA parameters (average path velocity, straight-line velocity and linearity)	Sørensen *et al.*[94]
	6·11(SD 29·19)	Healthy men (*n* 6)	Semen samples from spinal cord injured patients were obtained by electroejaculation	Hirsch *et al.*[135]
	2·35(SD 1·65)	Spinal cord injured (*n* 6)		
	4·17(SD 1·10)	Normozoospermic (*n* 19)	The total calcium concentration does not discriminate between fertile and infertile patients	Abou-Shakra *et al.*[99]
	5·04(SD 1·45)	Oligozoospermic (*n* 19)		
	4·47(SD 1·25)	Severely oligozoospermic (*n* 17)		
	4·87(SD 1·57)	Azoospermic (*n* 19)		
	6·11(SD 1·97)	Healthy men (*n* 22)	The total calcium concentration does not discriminate between fertile and infertile patients	Umeyama *et al.*[98]
	6·59(SD 3·12)	Infertile men (*n* 69)		
	7·50(SD 2·00)	Men at fertility clinical with normal semen parameters (*n* 17)	N.A.	Kavanagh[136]
	6·21(SD 1·55)	Normozoospermic (*n* 40)	N.A.	Adamopoulos and Deliyiannis[92]
	7·11(SD 0·37)	Azoospermic (*n* 12)		
Weighted average				
0·23	7·48			

CASA, computer-assisted semen analysis; CaSR, calcium-sensing receptor; *n*, cohort size; N.A., not applicable (the studies did not contain a conclusion on calcium levels and sperm function).
The ionised calcium and/or total calcium concentration measured in the seminal fluid or seminal plasma. The conclusions drawn by the authors of the papers regarding the calcium content and semen parameters are mentioned in the third column. All studies are mean(SD) except for Sørensen *et al.*[94], which is given as the median. The weighted averages are calculated based on the study sizes.

($[Ca^{2+}]_i$). An increase in $[Ca^{2+}]_i$ occurs by opening Ca^{2+} channels across the cell membrane or from intracellular stores – constituting a Ca^{2+} signal. $[Ca^{2+}]_i$ regulates capacitation, hyperactivation and the acrosome reaction[107]. Regulation of the $[Ca^{2+}]_i$ ensures the activation of spermatozoa at the appropriate time. The importance is indicated by studies showing that loss-of-function mutations in the gene coding for the main Ca^{2+} channel in sperm, CatSper, lead to infertility, and spermatozoa from men in fertility treatment have lower Ca^{2+} influx in response to progesterone compared with spermatozoa from healthy men[108,109]. Studies have shown that men

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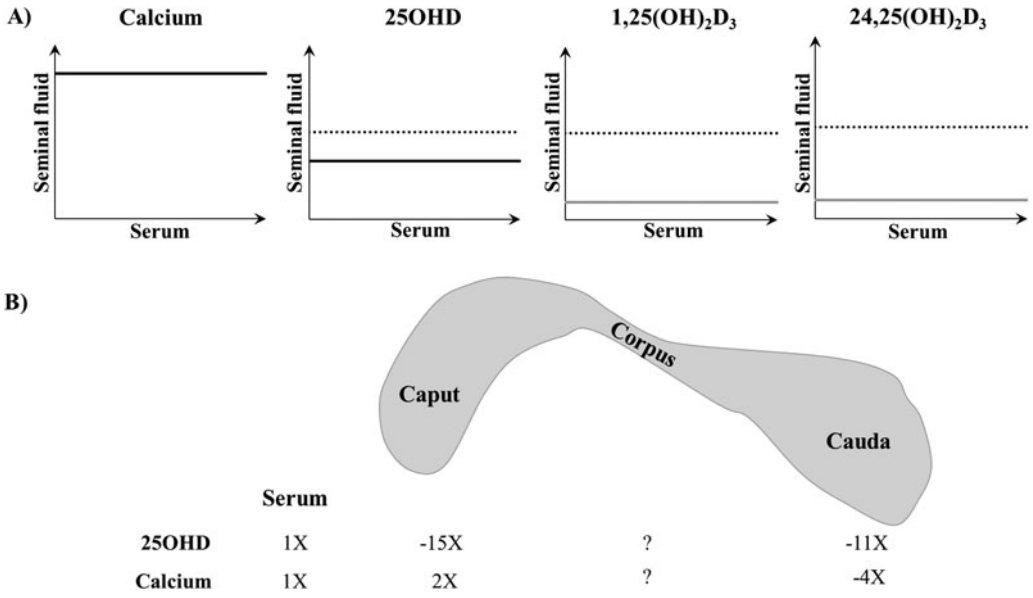

Fig. 1. Calcium and vitamin D in the male reproductive tract. (A) Seminal fluid concentration relative to serum concentration of calcium, 25-hydroxyvitamin D (25OHD), 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) and 24,25-dihydroxyvitamin D3 ($24,25(OH)_2D_3$) in men. Dotted lines represent the detection limit in seminal fluid, solid lines represent the constant concentration in seminal fluid and grey solid lines represent the undetectable levels in seminal fluid. The figure is based on data presented in[19,138]. (B) The concentration of calcium and vitamin D in the intraluminal fluid of rodent epididymis relative to the serum concentration. In caput, the 25OHD concentration is 15× lower and 11× lower in cauda compared with serum levels. The calcium concentration is 2× higher in caput and 4× lower in cauda. The figure is based on data presented in[76,101,102].

with severely impaired semen quality and men who had unsuccessful fertility treatment have significantly lower Ca^{2+} influx upon stimulation with female secreted factors compared with spermatozoa from healthy men indicating that these Ca^{2+} signals are clinically relevant[108,110].

Animal models with ablation of genes related to calcium homoeostasis in the testes or epididymis

Calcium homoeostasis in the male reproductive tract and its impact on male fertility has been studied in various animal models. Table 4 presents rodent models where a gene ablation has led to changes in local calcium homoeostasis in the testes or epididymis. The *Cyp27b1*$^{-/-}$ mice exhibit hypocalcaemia, decreased sperm count and motility resulting in impaired fertility. *Cyp27b1*$^{-/-}$ mice also have lower expression of calcium transport proteins including CaSR, CaV3·1 and TRPV5 in testes compared with wild-type mice[63]. Interestingly, a rescue diet (containing high calcium, phosphate and lactose) reestablished both the fertility and the expression of CaSR, CaV3·1 and TRPV5 in the testes of *Cyp27b1*$^{-/-}$ mice[63]. A study in *Vdr*$^{-/-}$ mice also found reduced *Casr* expression in the testes compared with wild-type mice[19], which is in accordance with the role of VDR as a regulator of calcium transporters in various organs. Previous studies have shown CaSR expression in the testes and spermatozoa from different species[63,111–117], and a proteomic study in bulls showed that the CaSR was the most differentially expressed protein when comparing good *v.* bad quality spermatozoa[118]. Moreover, treatment with different CaSR agonists increased sperm motility in rodents[111]. However, a germ cell-specific *Casr* knockdown model in male mice had no major reproductive phenotype compared with tamoxifen-treated controls irrespectively of the timing of *Casr* knockdown as conducted both pre- and post-pubertally (70 and 84 % reduction, respectively)[19], which questions the importance of CaSR in spermatozoa, at least in mice. In a recent paper investigating CaSR function in human sperm, three patients with loss-of-function mutations in *CASR*, and one patient with a gain-of-function mutation were included. Spermatozoa from all patients had aberrant Ca^{2+} signalling. Moreover, two of the patients with loss-of-function mutations in *CASR* had either low sperm motility and few morphologically normal spermatozoa, or calcifications in the efferent ducts[104].

Myotubularin-related protein 14 (MTMR14) is a phosphoinositide phosphatase and knockout of the gene results in impaired male fertility. In the *Mtmr14*$^{-/-}$ mice, the mRNA expression of the calcium channels inositol 1,4,5-trisphosphate receptor type 1 and 2 (*Itpr −1/−2*), and ryanodine receptor 3 (*Ryr3*) was decreased and $[Ca^{2+}]_i$ in spermatozoa derived from epididymis was lower compared with spermatozoa from wild-type mice[119]. Additionally, *Tmem203*-deficient male mice are sterile and exhibit a profound defect in spermatogenesis. mRNA expression profiling revealed down-regulation of the calcium channels *Trpv6* and transient receptor potential cation channel subfamily M members

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Table 4. Mouse knockout models that impact local calcium homoeostasis in the male reproductive tract

Approach	Subgroup	Fertility	Sperm count	Sperm motility	Sperm morphology	Impact on local calcium homoeostasis	Reference
Vdr KO	Black Swiss	N.D.	N.D.	N.D.	N.D.	Lower *Casr* expression in testes	Boisen *et al.*[19]
Cyp27b1 KO	BALB/c	↓	↓	↓	↓	Low expression of CaSR, CaV3·1 and TRPV5 in testes. Rescued by calcium-containing diet	Sun *et al.*[63]
Mtmr14 KO	C57BL/6	↓	↓	↓	↓	Decreased expression of the calcium channels *Itpr1*, *Itpr2* and *Ryr3* in spermatozoa. Spermatozoa had lower $[Ca^{2+}]_i$	Wen *et al.*[119]
Tmem203 KO	C57BL/6J	↓	↓	N.A.	↓	*Trpv6*, *Trpm5* and *Trpm8* were down-regulated in testes. *Pmca1* and *Ip3r1* were up-regulated. Altered calcium entry kinetics in testicular cells	Shambharkar *et al.*[120]
Sppl2c KO	C57BL/6N	→	N.D.	↓	→	SPPL2c cleaves phospholamban, which interacts with SERCA2 thereby regulating intracellular calcium concentration in germ cells	Niemeyer *et al.*[121]
Trpv6 KO	Mixed (129/C57BL)	↓	↓*	↓	N.D.	Calcium level in cauda epididymis was 10-fold higher resulting in low sperm motility	Weissgerber *et al.*[124]
Pmca4 KO	Mixed (129/black Swiss)	↓	N.D.	↓	→	PMCA4 is important in the regulation of basal calcium levels and calcium clearance in sperm	Schuh *et al.*[137]

CaSR, calcium-sensing receptor; *Ip3r1*, intracellular ion channel inositol 1,4,5-triphosphate receptor 1; *Itpr1/2*, inositol 1,4,5-trisphosphate receptor type 1/2; KO, knockout; *Mtmr14*, myotubularin related protein 14; N.A., not applicable; N.D., not determined; *Pmca1/4*, plasma membrane Ca^{2+} ATPase 1/4; *Ryr*, ryanodine receptor; SERCA2, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; *Sppl2c*, signal peptide peptidase-like 2C; *Trpm5/8*, transient receptor potential cation channel subfamily M 5/8; *Trpv5/6*, transient receptor potential vanilloid sub-type 5/6; *Vdr*, vitamin D receptor.
↓, negatively associated; →, no association; *the low caudal sperm count after swim-out may be explained by impaired motility rather than impaired sperm production or storage.

5 and 8 (*Trpm −5/−8*), and an increase in *Pmca1* and intracellular ion channel inositol 1,4,5-triphosphate receptor (*Ip3r1*) in testes. Testicular cells from *Tmem203* mice also exhibited altered calcium mobilisation[120]. Signal peptide peptidase-like 2C (*Sppl2c*) deficiency in male mice leads to dysregulation of the calcium pump sarcoendoplasmic reticulum calcium ATPase (SERCA2) involved in intracellular Ca^{2+} handling in male germ cells. The disturbed calcium homoeostasis resulted in impaired motility of spermatozoa but preserved fertility[121]. Efflux of intracellular Ca^{2+} can be mediated by the PMCA pump, which is essential for spermatozoa as *Pmca4*$^{-/-}$ mice are infertile due to Ca^{2+} overload and inability of spermatozoa to undergo capacitation[122,123].

The epididymis requires active calcium transport to maintain the calcium concentration gradient (Fig. 1B), which is necessary to ensure normal sperm function. In cauda epididymis in global *Trpv6* knockout mice a 10-fold higher calcium concentration impaired sperm motility[124]. Ma *et al.*[125] found an excessive calcium accumulation in the epididymis of vitamin K2-deficiency rats due to dysregulation of γ-glutamyl carboxylase (GGCX) and matrix gla protein (MGP). The vitamin K2-deficiency rats had lower sperm count and sperm motility. In agreement with the results in rats, the study also identified an SNP mutation in *GGCX* in an infertile man with asthenozoospermia, indicating that the influence of GGCX and MGP on fertility is conserved between species.

Human relevance of calcium in male fertility

The link between calcium, vitamin D, bone and gonadal function[126–129] complicates interpretations of human and animal studies as many of the effects of calcium may be indirect. In most human studies on vitamin D and male fertility, potential calcium-mediated effects have been neglected. In a study of infertile men, serum Ca^{2+} levels were negatively associated with sperm motility indicating that Ca^{2+} may influence semen quality in infertile men[10]. This association suggests that systemic regulators of calcium homoeostasis may also influence local calcium transport in the male reproductive tract, as calcium levels in serum and calcium levels in the epididymis and seminal fluid are not associated (Fig. 1A).

Significant interspecies differences exist in fertility and sperm function. Hence, extrapolating results from mice to human subjects in the fertility field is problematic and should be performed with caution[130]. Depending on the species, fertilisation occurs under entirely different aqueous conditions with different calcium and vitamin D levels, which remains to be thoroughly studied *in vivo*. Most data have been generated by *in vitro* studies and the true levels of calcium surrounding the spermatozoa as it

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moves through the female reproductive tract to the oocyte and in the moment of fertilisation are largely unknown.

Conclusions and perspectives

In conclusion, vitamin D and calcium are implicated in various facets of male fertility. Preliminary findings from intervention studies suggest that vitamin D supplementation may be beneficial for men with low vitamin D status, especially those with a serum 25OHD level below 25 nM/l. However, further investigation through larger clinical studies is required to enhance our understanding and address whether vitamin D supplementation can improve semen quality mostly in men with insufficient vitamin D levels. It seems that many of the observed effects of vitamin D are mediated indirectly through changes in local levels of factors such as calcium or phosphate, not only in the testes but also in epididymis, prostate and seminal vesicles. The crucial role of calcium in male reproductive function extends beyond sperm motility and fertilisation, as calcium signalling is involved in various processes, such as spermatogenesis, sperm maturation, capacitation, acrosome reaction and imbalances in calcium levels may disrupt these events and impair fertility. The exact mechanisms underlying the impact of calcium on male reproduction are not fully understood, although studies have suggested that maintaining optimal calcium homoeostasis is essential for overall reproductive health.

More research on the specific role of vitamin D and other regulators of local calcium and phosphate signalling in the male reproductive organs is warranted. Recently, we identified the receptor activator of NFκB ligand as a novel regulator of the production and maturation of spermatozoa[128]. Inhibition of receptor activator of NFκB ligand increased sperm motility and sperm count in a subgroup of men with preserved Sertoli cell capacity, and identification of predictive biomarkers for positive treatment outcomes is crucial and requires further investigation. Moreover, it is crucial to shift focus towards investigating more definitive outcomes such as conception rates and live births. Currently, there is a concerning lack of evidence-based treatments available for men with idiopathic infertility, despite the high prevalence of male infertility[7,131]. Rather than tailoring treatments to the specific causes of infertility[17,24,129,132], most infertile couples are commonly subjected to inseminations or assisted reproductive techniques. Assisted reproductive techniques have demonstrated high success rates, but they come with substantial financial costs and impose a significant burden on the female partner. The invasive procedures and prolonged hormonal treatments often lasting several months contribute to the treatment burden faced by women undergoing assisted reproductive techniques. In this regard, supplementation of vitamin D presents a promising avenue for improving semen quality in certain cases of idiopathic male infertility and supports screening of vitamin D and mineral status in cases of idiopathic male infertility. It offers a safe and non-invasive treatment option that could potentially benefit some infertile couples. Moreover, the devastating effect of vitamin D deficiency on semen quality in animal models and human subjects indicates that society should continue to focus on preventing people from having vitamin D deficiency.

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Conflict of Interest

M. B. J. holds two patents on the use of RANKL inhibitors to treat male infertility. All other authors state no conflict of interest.

Authorship

S. K. Y. and I. M. B. reviewed the literature, wrote the initial draft and had the main responsibility for the preparation of the paper. M. B. J. was responsible for the overall paper design, critical discussions and final approval of the manuscript. In line with the mentioned authors, Z. C., M. J. J., I. K., R. H. and A. J. participated in the discussion and interpretation of the results, critically revised the manuscript for intellectual content and approved the final version.

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