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Review Article

Zinc: Crucial Ion for Male Fertility in the *In vitro* Reproduction Era

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Abstract

The importance of zinc ion in male fertility was recently proposed in several studies. In the present review we describe the properties, roles and cellular mechanisms of action of Zn^{2+} in spermatozoa. We focused on the involvement of zinc ion in sperm motility, capacitation and acrosomal exocytosis, three functions that are crucial for successful fertilization. The impact of zinc supplementation on fertilization assisted techniques is also described.

Abbreviations

AC: Adenylyl Cyclase
sAC: soluble AC
tmAC: trans membrane AC
AE: Acrosomal Exocytosis
EGFR: Epidermal Growth Factor Receptor
ICSI: Intra Cytoplasmis Sperm Injection
GPCR: G-protein Coupled Receptor
HAM: Hyper Activated Motility
IP3: Inositol Triphosphate
IVF: *In Vitro* Fertilization
NHE: Na^+/H^+-exchanger
ODF: Outer Dense Fibers
PI3K: Phosphatidylinositol-3-Kinase
PKA: Protein Kinase A
PLC: Phospholipase C
PLD: Phospholipase D
ROS: Reactive Oxygen Species
ZnOPs: Zn Oxide Particles
ZnR: Zn Receptor
ZP: Zona Pellucida

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Introduction

Zinc is an essential element for many biological activities including enzyme regulation, mitochondrial oxidative stress, normal growth, spermatogenesis, digestion and regulation of central nervous system [1]. The concentration of zinc in the body is precisely regulated, and imbalance of zinc would accompany several pathologies including Alzheimer [2-4], cancer, growth retardation, blindness, digestive problems and inflammation [5]. In human body, about 90% of Zn^{2+} is found in bone and muscle [6]. About 0.1% of bodily Zn^{2+} is in the serum 60% of it bound to albumin and the remaining to other proteins [7-9]. On the tissue level, 30-40% of Zn^{2+} is found in the nucleus, 50% in the cytoplasm and the rest in cell membranes [10]. The concentration of Zn^{2+} in the blood is 3.14mg/l or 4.8mM [11] and in semen ~2mM which is positively correlated with sperm count and normal morphology [12]. Zn^{2+} deficiency triggers autophagy in yeast [13], which would affect spermatogenesis. It has been shown that Zn^{2+} supplementation improves serum testosterone levels [14], sperm count [15], plasma membrane and acrosome integrity [16] and restores superoxide antioxidant capacity in asthenospermic men [17]. Interestingly, Zn^{2+} improves intestinal epithelial barrier function [18] and the integrity of mammary epithelium [19]. Thus it is possible that Zn^{2+} might enhance the integrity of the epithelium in the capacitation site in the female reproductive tract, resulting in improving sperm fertility. Too high Zn^{2+} in the dietary to both male and female rats shows significant reduction in fertility [20]. Zn^{2+} has been linked with key events in the accomplishment of fertilization ability including hyperactivation and acrosomal exocytosis. Defects in sperm quantity, quality and motility account for up to 50% of infertility cases and may affect about 7% of all men [21]. About 25% of infertility cases in human are defined as “unexplained infertility”, and in many cases, a successful fertilization in these men can be achieved by Intra-Cytoplasmic-Sperm-Injection (ICSI) technique. On the other hand, in a not negligible part of these unexplained cases, despite normal sperm quantity, morphology and motility, no egg penetration/fertilization occurs. It is well documented that in order to fertilize, sperm should reside in the female reproductive tract for several hours, in which they undergo a series of biochemical and motility changes collectively called capacitation allowing the spermatozoon to interact with the egg, undergo acrosomal exocytosis and penetration into the egg. Thus it is possible that a significant part of unexplained infertility, that have not been resolved by bypass techniques like ICSI, are in fact caused by spermatozoon failure to performing proper capacitation.

It was shown that zinc deficiency is correlated with a decrease in male fertility [22,23] and zinc in the dietary of dometic animals is required for the achievement of higher fertility rate [24-26]. Sperm mitochondrial sheath [27,28] and sperm chromatin [29,30] are stabilized by zinc bridges. In this review we will focus on the effect of Zn^{2+} on sperm capacitation, acrosomal exocytosis, including the mechanisms of action and the impact of zinc supplementation on fertilization assisted techniques.

Regulation of Zn^{2+} Levels in the Cells

Research in *C. elegans* identified many genes that were defective in spermatogenesis (spe) and fertilization when mutated [31,32].

For example, spe-8gene that encodes protein tyrosine kinase, involved in protein tyrosine phosphorylation [33,34], a known process that occurs in sperm capacitation [35]. Several proteins function with SPE-8, mediating signaling pathways that promote motility [36,37]. It has been suggested that zinc may initiate SPE-8 signaling cascade leading to sperm activation [38,39]. Working on zinc-transporters revealed that deletion of the homolog zipt-7.1, caused sterility [40]. Zipt-7.1 is a transmembrane protein localized within intracellular organelles [41,42] and together with SPE-8 regulates the release of Zn^{2+} from internal stores. The released Zn^{2+} in the cytoplasm activates zinc-regulated proteins that develop motility. Thus, Zn^{2+} may be considered as second messenger which modulates sperm functions like motility and capacitation. This suggests that intracellular Zn^{2+} levels should be well controlled by zinc transporters localized in intracellular membranes and in the cell plasma membrane which import Zn^{2+} from external environment [43].

Effect of Zn^{2+} on Sperm Capacitation and Acrosomal Exocytosis

Extracellular zinc had an impact on the intracellular signaling pathway via its interaction with the Zinc Sensing Receptor (ZnR), named also GPR39 [44]. This receptor was found in the sperm acrosome and tail [45-47] suggesting a possible involvement of zinc in sperm functions. We showed that Zn^{2+} stimulates bovine sperm acrosomal exocytosis [45] as well as Human sperm Hyperactivated Motility (HAM) [46] both mediated by GPR39. The GPR39 belong to GPCR family known to activate the Trans-Membrane-Adenylyl-Cyclase (tmAC). Human sperm treated with 5μM Zn^{2+} show a 40% increase in intracellular cAMP which is an important event in the capacitation process [45]. It seems that zinc mediates the activity of the two AC isoforms, the sAC as well as the tmAC, leading to intracellular cAMP increase; an effect that was inhibited by the respective specific proteins inhibitors. Surprisingly, the stimulatory effect of extracellular added 8Br-cAMP (a membrane permeabile cAMP analogue) on HAM was also inhibited by sAC inhibitors, conditions by which the cellular levels of cAMP should not be affected [46]. A possible explanation for this result would be that cAMP supplied to the cells is excluded from cellular locations in which sAC provides cAMP for HAM. Interestingly, attempts to bypass the need for sAC activity by providing cAMP did not restore fertilization competence of sAC-null sperm [48]. It has been shown that in vitro addition of high concentration of $Zn^{2+}$to bovine [45] and human [46] sperm could lead to the inhibition of several capacitation processes and fertility rate [49]. Zinc has antioxidant activity and may decrease Reactive-Oxygen-Species (ROS) levels [16,50]. It was shown that ROS production is essential for sperm capacitation [51,52] however relatively high levels of ROS can harm sperm functions [53]. Thus low zinc concentration might be beneficial in reducing too high levels of ROS, whereas high zinc might decrease ROS to a level that is inhibitory to sperm capacitation. A relatively high concentration of Zn^{2+} in the millimolar range inhibits human sperm motility [54] and regulates the degradation of semenogelin that prevents capacitation via inhibition of ROS generation [46,55]. This high [Zn^{2+}] also inhibits the voltage-gated H^+-channel Hv1, localize in sperm tail and responsible for sperm cytoplasmic alkalization [56,57] and the regulation of human sperm rotation [58]. The cytoplasmic alkalization leads to the activation of the sperm-specific Ca^{2+}-channel CatSper [59] which mediates the development of the capacitation dependent HAM [60]. The stimulatory effect of Zn^{2+} on human sperm HAM is inhibited by CatSper inhibitor indicating that CatSper mediates Zn^{2+}-stimulated HAM [46]. Zinc enhanced Protein-Kinase A (PKA) activity, Src and Epidermal-Growth-Factor Receptor (EGFR) phosphorylation/activation and these activities are CatSper-dependent [46]. It was shown that Zn^{2+} is incorporated into sperm ODF extending from the connecting piece of the tail, causing softening of its consistency leading to the development of HAM [61]. In contradiction to the high seminal fluid [Zn^{2+}] (~2mM) which inhibits Hv1, lower concentrations in the micromolar range promote acrosomal reaction in sea urchin [44] and bovine [45] sperm. In sea urchin sperm micromolar Zn^{2+} activates changes in membrane potential, induce elevation of pHi,[Ca^{2+}] iand cAMP and activate K^+-channel [62].

Also, we found that 5-10μM Zn^{2+} stimulates hyperactivated motility in human sperm incubated under capacitation conditions, whereas at 30μM Zn^{2+} there is no stimulation [46]. These data clearly show that the relatively high [Zn^{2+}] in the semen are inhibitory to sperm functions, whereas in the female reproductive tract [Zn^{2+}] is much lower (1.0-1.5μM) [63] allowing the occurrence of sperm capacitation/hyperactivated motility and the acrosome reaction leading to fertilization. It has been proposed [64] that Zona Pellucid (ZP) proteinases implicated in endowing the acrosome reacted spermatozoon with the ability to penetrate the ZP, are negatively regulated by Zn^{2+}. It has been shown that sperm can induce Zn^{2+} release from the oocyte cortex [65,66] leading to proteinases inhibition and as a result sperm that are still bound to the ZP became de-capacitated, and polyspermy is prevented. It was also suggested that Zn^{2+} inhibits sperm chemoattraction to the egg induced by oocyte-secreted progesterone in human, mouse and rabbit sperm [67]. Addition of Zn^{2+} (~0.1mM) to bovine IVF medium inhibits fertilization rate [68]. Also, blockers of Zn-dependent metalloproteases inhibit sperm passage via the cumulus ooporus in porcine IVF [69].

Appropriate concentration of zinc, in the micromolar range, seems to increase in vitro capacitation efficiency [45,46] by activating several proteins during this process, including the tyrosine kinase Src, EGFR transactivation and Phosphatidylinositol-3-Kinase (PI3K) [45,70-73] leading to Ca^{2+} mobilization and acrosome reaction. In a recent study, we suggested the following mechanism that regulates human HAM: Zn^{2+} stimulates HAM via CatSper-dependent activation of the Adenylyl-Cyclase (AC)/cAMP/PKA/Src/EGFR and Phospholipase C (PLC) cascade [46]. In bovine sperm, we show that Zn^{2+} activates the EGFR during capacitation which is mediated by activation of tmAC, PKA and Src [45]. The addition of Zn^{2+} to capacitated bovine sperm further stimulates EGFR and the down-stream effectors PI3K, phospholipase C and protein-kinase C leading to acrosomal exocytosis [45].

Zinc in Assisted Reproductive Techniques

Over the past decade, the efficiency of assisted reproductive techniques has been improved. The cryopreservation of sperm using liquid nitrogen is now usually used in assisted reproduction centers and laboratories as a procedure to preserve sperm cells. However, freezing and thawing processes, cause a decrease of the fertilizing sperm efficiency due to various stress and cryoprotectant toxicity. The sperm is deprived of the seminal plasma protective effects; many antioxidants are stored in human seminal plasma such as vitamin c and e, superoxide dismutase, glutathione and thioredoxin that act directly against free radicals [74,75]. It is well known today that osmotic effects and oxidative stress of cryopreservation affect sperm cells in many ways: by diminishing fertilization capacity, motility, morphology

(such as coiled tails), viability of spermatozoa [76] damaging cells membrane [77] causing DNA fragmentation [78,79] and loss of mitochondrial function [80]. The improvement of fertility capacity by certain antioxidants has been more and more used in assisted reproduction techniques [81,82]. The addition of zinc to the culture medium was reported to protect the human spermatozoa from oxidative damage [83]. Studies revealed that after incubation with H_20_2 the DNA fragmentation percentage in spermatozoa was increased (in comparison to control), effect that was reversed by zinc supplementation to the medium [83].

Recent researches brought to light the beneficial effects of zinc addition to human ejaculate before cryopreservation on sperm viability and motility after thawing [83,84]. Freezing of human sperm in the presence of 50µM zinc revealed after thawing a 26%-184% increase in the number of motile sperm and a 130 % increase in the percentage of progressive motility [84]. Similar effects were observed in semen samples cryopreserved with Zinc Oxide Nanoparticles (ZnONPs) after thawing and followed by an incubation of 24h [85]. Moreover when cells were frozen, thawed and refrozen in a second time in the presence of zinc a considerable increase in motility was observed [84]. These significant improvements in sperm motility, when zinc was supplemented to cryopreservation media can be associated with the ion effects on microfilament in the outer dense fiber [86], leading to an increase in sperm mobility percentage [87]. Moreover, zinc has been reported to preserve genomic integrity [88], chromosomal stability [89,90], and protect sperm membrane [91,92] preserving in that way cell morphology during cryopreservation.

Utilization of ZnONPs, used basically as drug delivery for cancer research [93], was applied to study sperm preservation cells during cryopreservation. The ZnONPs seemed to provide beneficial effects by avoiding DNA damages and by stabilizing sperm chromatin [85]. These protecting effects were reported to be linked to the creation of a protective layer of ZnONPs around the sperm cell preventing lipid peroxidation at the membrane [85]. Considerable IVF cases rise from male-factor deficiencies. The quality of sperm after cryopreservation is an essential factor in the success of assisted reproduction procedures. Zinc can be considerate as a good player to this issue (Figure 1).

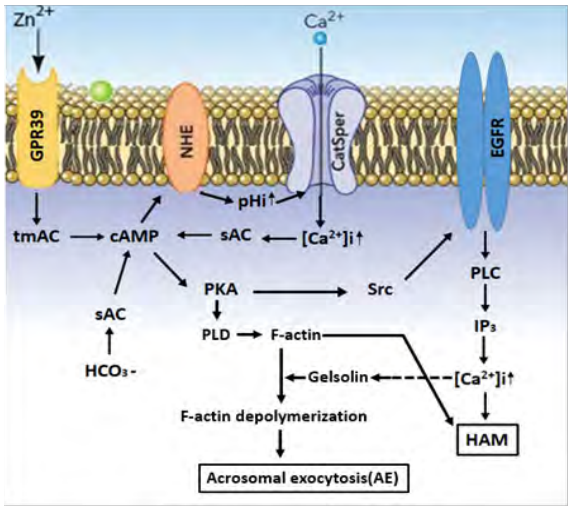

Figure 1: A model describing the mechanisms that mediate the stimulation of HAM and Acrosomal Exocytosis (AE) by Zn^{2+}.

Zn^{2+} binds and activates GPR39 which activates the tmAC to catalyse cAMP production. NHE (Na^+/H^+-exchanger) is activated by cAMP leading in increase pHi and activation of CatSper resulting in an increase in $[Ca^{2+}]$ i which together with HCO_3^- activates sAC. The increase in [cAMP] i causes PKA-activation following by activation of the cascade Src-EGFR-PLC resulting in IP3 production which mobilizes Ca^{2+} from the acrosome causing further increase in $[Ca^{2+}]$ i and the development of hyper-activated motility. PKA also activates PLD leading to F-actin formation during capacitation. Prior to the AE, Ca^{2+} activates the actin severing protein gelsolin resulting in F-actin depolymerization and Acrosomal Exocytosis (AE).

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