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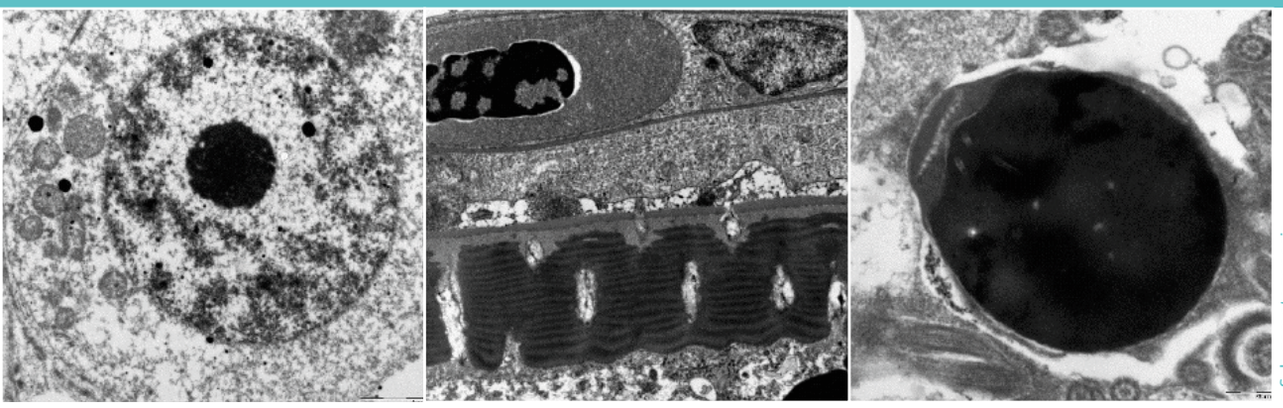
Regulation of subcellular calcium during fish gametogenesis

Regulace subcelulárního vápníku během gametogeneze ryb



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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Calcium is a ubiquitous element in the living systems that regulates different vital biological processes such as heart beat, neurotransmission, reproduction and etc. Several mechanisms inside the cell compartment regulate calcium. There are major types of proteins directly involved in calcium homeostasis including calcium binding proteins, channels, ATPases and exchangers. Calcium binding proteins such as calsequestrin are located in endoplasmic reticulum and act as dynamic storage molecules for low affinity, high capacity calcium storage. A calcium channel is an ion channel that specialized for selective permeability to calcium ion and allows facilitated diffusion of calcium down its concentration gradient. However, calcium pumps possess the ability to transport calcium against a concentration gradient in an energy-dependent way. Exchangers such as the Na^{+}/Ca^{2+} exchanger, utilize the energy that is stored in the electrochemical gradient of sodium by allowing three Na+ ions to flow down its gradient across the plasma membrane into the cell in exchange for the transport of one Ca^{2+} out of the cell (Monteith et al., 2007).

Calcium signals are important for life, but any disorder in calcium homeostasis can alter signals of life to signals of death (Berridge et al., 1998). The concentration of free calcium in the cytoplasm of resting eukaryotic cells is estimated to be around 10000 times lower than in the extracellular environment where it is present in higher concentrations. This huge gradient is maintained by actively extruding calcium from the cytosol, either by pumping it out of the cell or into intracellular storage compartments such as endoplasmic reticulum and mitochondria. Although calcium homeostasis is very energy consuming for the cell, it is essential for normal survival and development; prolonged high free calcium levels are normally lethal for most cells, leading to apoptosis or necrosis (Orrenius et al., 2003).

Roles of calcium in fish spermatogenesis

Spermatogenesis is a fundamental process during which a small number of diploid spermatogonial stem cells produce a large number of highly differentiated spermatozoa carrying a haploid, recombined genome (Schulz et al., 2010). Based on morphological and physiological studies, spermatogenic cells are categorized as spermatogonium, spermatocyte, spermatid and the spermatozoon (Wallace, 1985; Selman et al., 1993; Schulz et al., 2010). In fishes, spermatogenesis is initiated with the mitotic proliferation of spermatogonia, proceeds through two meiotic divisions, and concludes with spermiogenesis, during which the haploid spermatids transform into motile spermatozoa (Schulz et al., 2010).

The ultrastructural features of a spermatogonium is categorized as the largest germ cell type in the seminiferous tubules. These cells are identified as slightly oval or spherical in shape with a single prominent nucleus and a nucleolus. Intracellular calcium has been found in the rat spermatogonium as deposit form (Ravindranath et al., 1994). This evidence has emphasized importance of calcium in regulation of vital biological events in these cell types (Ravindranath et al., 1994). It has been reported that calcium likely contributes ton the regulation of the spermatogonial transition from a proliferating phase to meiosis (Chiarella et al., 2004).

The ultrastructure of spermatocytes in fish testis is illustrated with round nucleus with evenly dispersed chromatin and synaptonemal complexes (Leal et al., 2009). Among the population of germ cells, spermatocytes contain the lowest amount of calcium which steadily increases in subsequent stages of germ cell differentiation (Santi et al., 1998). Similar to spermatogonia, intracellular calcium has been shown to be accumulated in a deposit form in the nucleus and cytoplasm of spermatocyte (Ravindranath et al., 1994; Feng et al., 2006). This event is further supported by study on Crane fly spermatocytes that shows calcium is

not free, but rather is sequestered (Forer et al., 1980). Significant elevation of calcium from spermatogonia to later stages of spermatogenesis has been evident in rat seminiferous tubules during spermatogenesis (Hagiwara and Kawa, 1984).

Ultrastructural features of a fish spermatid cell is defined by a round nucleus with granular chromatin and considerable cytoplasm, and round or elliptical shaped mitochondria with an electron-dense matrix (Leal et al., 2009). Experimental studies have demonstrated that calcium is essential for the spermatid differentiation (Ravindranath et al., 1994; Feng et al., 2006). However, the morphological importance of intracellular calcium in spermatid cells is not yet known.

Ultrastructurally, the spermatozoon has a spherical, homogeneously electron-dense nucleus and a short midpiece containing spherical mitochondria that encircles the basal body of the flagellum (Quagio-Grassiotto et al., 2003). Numerous studies have described that intracellular calcium is distributed in the plasma membrane, acrosome membrane, and the mitochondrial matrix (Ravindranath et al., 1994; Feng et al., 2006). All of the studies that have been done so far, provide direct evidence regarding involvement of cytoplasmic calcium stores to control many physiological functions such as motility (Sakata et al., 2002), capacitation and hyperactivity (Breitbart, 2002; Suarez and Ho, 2003), and acrosome reaction (Feng et al., 2006).

Roles of calcium in fish oogenesis

Oogenesis is a fundamental and convoluted process in which the female haploid gamete (egg) is developed and differentiated through meiotic divisions (Lubzens et al., 2010). According to the available literature, oocyte development during oogenesis is divided into four stages in the teleost species, based on morphological criteria and on physiological and biochemical events (Selman et al., 1993). However, oocyte development has been observed to be slightly different among species. Although oocyte maturation has been studied in a variety of vertebrates, the distribution and potential roles of intracellular calcium during different developmental stages of oogenesis have not been yet addressed in fishes, comprehensively.

The first stage is identified as primary growth and divided into two or three phases. In this stage, cells are typically characterized by a large nucleus relative to the volume of cytoplasm, and peripherally located nucleoli. With regard to this stage, many studies on mammalian species have reported the distribution of intracellular calcium deposits within the oocytes. These deposits are mainly observed in the nucleus, cytoplasm and granulosa cells surrounding the oocyte (Petr et al., 1997; Sedmikova et al., 2003; Rozinek et al., 2006). The possible role of intracellular calcium deposits in the nucleus can be explained by presence of numerous calcium-binding proteins within the nucleus that assist accumulation of higher amounts of calcium ions for protein synthesis (Bachs et al., 1994; Gilchrist et al., 1994). It is also interesting to note that accumulation of calcium deposits within organelles in the oocyte is not surprising as it correlates with the calcium distribution in other types of cells (Sedmikova et al., 2003). Hence, it has been proposed that, the presence of calcium in the granulosa cells might be due to its involvement in processes occurring in the somatic compartment of the follicle (Lebedeva et al., 1998; Jayes et al., 2000).

The second stage is cortical alveolus stage which is characterized by the appearance of cortical alveoli within the oocyte (Selman et al., 1993). The cortical alveoli are specialized membrane-limited secretory organelles, which play an important role in the morpho-functional processes produced after the fertilization. Studies have emphasized the cortical alveolus contains high amounts of calcium compared with other compartments that may further involved in cortical reaction process (Schuel, 1978; Gillot et al., 1991). This fact is

underlined by the considerable role of calcium in formation and hardening of the egg envelope in echinoderms and amphibians (Runnstrom, 1966; Wolf, 1974). It has been thought that calcium stores in cortical granules stabilize the structure of the membrane or possibly provide the source of the intracellular calcium that is released at fertilization to initiate the cortical reaction and activate development (Schuel, 1978).

The third stage of oocyte development is known as vetillogenesis which is evident by the increase in the size of oocyte due to presence of yolk bodies and clear appearance of zona radiata layers (Wallace and Selman, 1985; Selman et al., 1993). Several metabolic changes, such as an increased level of calcium occur during vitellogenin synthesis in oocytes of female fish. Correspond to this stage in a fish oocyte, calcium deposits fluctuate significantly and are distributed in different organelles. This likely can be attributed to the role of calcium in acquisition of oocyte growth and follicular development with regard to its content in various cellular compartments (Sedmikova et al., 2003; Rozinek et al., 2006).

The late stage of oocyte development is defined as maturation in which meiosis is reinitiated and the germinal vesicle migrates toward the oocyte periphery (Wallace and Selman, 1985; Selman et al., 1993). Most observations in this stage through studies in different animals, indicate storage of calcium deposits in the mitochondria which has a significant role in the regulation of meiosis resumption (Liu et al., 2001; Dumollard et al., 2004, 2006).

Roles of calcium in egg activation and fertilization in fish

During fertilization in animals, each egg must produce a proper intracellular calcium signal for development to proceed normally. This hypothesis suggests that changes in the concentration of calcium ions in the ooplasm contribute in oocyte activation (Loeb, 1899). The role of calcium as the trigger of oocyte activation was proven when in medaka oocyte, fertilization was shown to be accompanied by an elevation in the intracellular free calcium concentration (Ridgway et al., 1977). In sea urchin, inhibition of this elevation is accompanied with pause of activation (Zucker and Steinhardt, 1978; Whitaker and Steinhardt, 1982). Studies on a wide variety of vertebrate eggs have indicated that development at fertilization is activated by an elevation in intracellular calcium concentration (Coward et al., 2002, 2005). This elevation has been shown to occur due to repeated release and re-uptake of calcium stores from different organelles within the egg, particularly endoplasmic reticulum (Stricker, 1999; Machaty, 2016). This alteration in intracellular calcium activates the egg and also appears to act as key factor in later embryonic development.

In most species, the spermatozoon have a significant role in triggering a single elevation in the egg's intracellular free calcium concentration. This phenomena commences at the site of the spermatozoon entry and travels across the oocyte as a propagating calcium wave (Gilkey et al., 1978). The mechanism that explains how the spermatozoon triggers the calcium elevation in the ooplasm remained unknown for decades. A few hypotheses were proposed to clarify the generation of the fertilization calcium signal. At first the "calcium bomb hypothesis" claimed that the spermatozoon injects calcium into the oocyte that forms a wave of calcium-induced calcium release (Jaffe, 1983). However, this hypothesis was modified later to suggest that the spermatozoon serves as a calcium stimulator, allowing calcium transfer from the extracellular medium to dessiminate into the ooplasm (Jaffe, 1991). Subsequently, calcium is accumulated in the endoplasmic reticulum through pumping calcium, which results in the overloading of the stores and the release of luminal calcium.

Another hypothesis is called "receptor hypothesis" in which the fertilizing spermatozoon is considered as main factor to induce the calcium fluctuation by binding to a receptor on the surface of the oocyte plasma membrane. This hypothesis along with supported findings for

a long time has been accepted as the prime model to explain generation of the fertilization calcium signal. However, numerous publications have established a lack of evidence that the spermatozoon triggers oocyte activation via these pathways (Jaffe, 1990; Schultz and Kopf, 1995). To support this, Wassarman et al. (2005) demonstrated no role of ligands and receptors characterized on the surface of mammalian gametes which involved in the mediation of sperm-egg binding and fusion.

The last assumption known as "the spermatozoon factor hypothesis", proposes the contribution of a soluble factor in the spermatozoon which induces oocyte activation. This theory was described in numerous publications associated with fusion between the spermatozoon and egg membranes that triggers the first calcium transient wave in a short period. It is consistent with the sense that the spermatozoon-resident factor requires time to move into the oocyte cytoplasm before calcium deliver from the internal stores (Lawrence et al., 1997). This hypothesis has further been evaluated by injection of a crude extract isolated from the head of mammalian spermatozoon which was able to induce repetitive calcium oscillations in mammalian oocytes (Swann, 1990; Wu et al., 1997; Machaty et al., 2000). In fish and frogs, sperm extracts have successfully induced calcium oscillations in the mouse oocyte (Dong et al., 2000; Coward et al., 2003).

Objectives of the thesis

We designed several experiments to investigate how cells in different developmental stages of gametogenesis manage to keep calcium homeostasis to maintain proper cell function. We used an ultracytochemical technique followed by a software analysis to localize and quantify subcellular calcium in the different developmental stages of gametogenesis in fish. We tried to find links between regulations of calcium dynamics in subcellular levels with the possible role of calcium in cell functions during different developmental stages of gametogenesis in fish. Therefore, the aims of present study were to:

1. Ultrastructural localization and quantification of subcellular calcium during different developmental stages of spermatogenesis in sterlet (*Acipenser ruthenus*).
2. Ultrastructural localization and quantification of subcellular calcium in zebrafish (*Danio rerio*) spermatogenesis
3. Ultrastructural localization and quantification of subcellular calcium in zebrafish (*Danio rerio*) oogenesis.

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CHAPTER 2

ULTRASTRUCTURAL LOCALIZATION OF INTRACELLULAR CALCIUM DURING SPERMATOGENESIS OF STERLET (*ACIPENSER RUTHENUS*)

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MICROGRAPHIA

Ultrastructural Localization of Intracellular Calcium During Spermatogenesis of Sterlet (*Acipenser ruthenus*)

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Abstract: Calcium regulates many intracellular events such as growth and differentiation during different stages of gamete development. The aim of this study was to localize and quantify the intracellular distribution of calcium during different developmental stages of spermatogenesis in sterlet, *Acipenser ruthenus*, using a combined oxalate–pyroantimonate technique. The distribution of calcium was described in spermatogonium, spermatocyte, spermatid, and spermatozoon stages. In the spermatogonium and spermatocyte, calcium deposits were mainly localized in the nucleus and cytoplasm. The spermatid had calcium in the nucleus, developing acrosomal vesicle, and cytoplasm. Intracellular calcium transformed from scattered deposits in spermatogonia and spermatocyte stages into an unbound form in spermatid and the spermatozoon. The proportion of area covered by calcium increased significantly ($p < 0.05$) from early to late stages of spermatogenesis. The largest proportion of area covered by calcium was observed in the nucleus of the spermatozoon. In conclusion, although most of the intracellular calcium is deposited in limited areas of the spermatogonium and spermatocyte, it is present an unbound form in the larger area of spermatids and spermatozoa which probably reflects changes in its physiological function and homeostasis during the process of male gamete production in spermatogenesis.

Key words: calcium, oxalate–pyroantimonate, spermatozoon, subcellular localization

Introduction

Spermatogenesis is a developmental process during which a small number of diploid spermatogonial stem cells produce a large number of highly differentiated spermatozoa carrying a haploid, recombined genome (Schulz et al., 2010). Ultrastructure studies on spermatogenesis and spermatozoa exhibit wide ranges of structural variations among species that could be valuable to enhance understanding of germ cell differentiation (Mattei, 1991; Biagi et al., 2015).

The importance of calcium in reproduction has been demonstrated by its predominant role in the regulation of many functional processes of spermatogenesis and fertilization (Ravindranath et al., 1994; Feng et al., 2006; Lee et al., 2011). Studies have proven that calcium signals are present not only in somatic cells but also in the germ cells (Darszon et al., 1999; Stricker, 1999). During spermatogenesis, intracellular calcium presumably signals different specific cellular functions (Treviño et al., 1998; Chiarella et al., 2004). Numerous calcium channels have been found in spermatogenic cells and spermatozoa which likely are involved in the control of calcium signals (Walensky & Snyder, 1995; Santi et al., 1996; Serranoa et al., 1999). Calcium is involved in important biological processes such as growth, proliferation and cell death in the spermatogonium and spermatocyte cells (Ravindranath et al., 1994; Treviño et al., 1998). In addition, several key functions in spermatozoa including motility (Sakata et al., 2002), capacitation and hyperactivity (Breitbart, 2002; Suarez & Ho, 2003), and acrosome reaction (Feng et al., 2007; Pšenička et al., 2009) are regulated by cytoplasmic calcium.

Subcellular localization of calcium during different developmental stages of gametogenesis in rat (Ravindranath et al., 1994), pig (Petr et al., 2001; Rozinek et al., 2006), mouse (Sedmiâkovâ et al., 2003), and zebrafish (Golpour et al., 2016) using a combined oxalate–pyroantimonate technique have provided useful information regarding involvement of calcium in the basic processes of reproduction. Despite the importance of calcium in cellular events, and considering that fishes make up more than half of all vertebrates, there is no data available on the distribution of intracellular calcium during spermatogenesis in fish.

The sterlet, *Acipenser ruthenus*, is a widely distributed freshwater sturgeon in east-central Europe and Asia. It is commercially important for caviar and biological studies (Sokolov & Vasilev, 1989). Their wild populations have decreased due to overfishing for caviar, poaching, and environmental pollution but are now being replenished in some parts of the world through artificial reproduction and annual stocking programs (Williot et al., 2001). Therefore, fundamental knowledge about reproductive biology of

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sturgeon may improve the efficiency of artificial reproduction. Studying the ultrastructural distribution of calcium in spermatogenesis can provide some clues about calcium homeostasis by cells and other possible roles in different stages of male gamete development.

The purpose of the present study was to localize and compare the ultrastructural distribution of intracellular calcium among the different developmental stages of spermatogenesis in sterlet *A. ruthenus*.

Materials and Methods

Cultivated sterlet broodstock (3–5 years old, weight 0.76–1.18 kg) were provided by the Fischzucht Rhönforelle GmbH & Co. KG, Marjoss, Germany. Fish were kept at the hatchery of the Research Institute of Fish Culture and Hydrobiology during the natural spawning season in outdoor 4 m^3 plastic tanks with a 20 l/min pond water inflow at 8–12°C.

Three males were sampled in each of the four seasons (n = 12). Fish were anesthetized, killed, and gonads were removed, cut, and immersed immediately in the primary fixative solution (2% glutaraldehyde, 2% formaldehyde, 90 mM potassium oxalate, and 1.4% sucrose, pH 7.4 adjusted by KOH), at room temperature and then held at 4°C for 48 h. The tissues were washed three times, 15 min each, in distilled water and then post-fixed in 1% osmium tetroxide and 2% potassium pyroantimonate for 3 h at 4°C (Niksirat & Kouba, 2016). Then, samples were rinsed thoroughly three times, 15 min each, in cold distilled water with pH raised to 10 using KOH (Ravindranath et al., 1994; Golpour et al., 2016). Samples were dehydrated in an increasing acetone series and embedded in resin (EPON). A series of ultrathin sections were cut using a UCT Ultramicrotome (Leica Microsystems, Wetzlar, Germany) mounted on copper grids, double-stained with uranyl acetate and lead citrate for contrast, and examined with a 1010 transmission electron microscope (TEM) (JEOL Ltd., Tokyo, Japan) operating at 80 kV (Niksirat et al., 2015). For better detection of intracellular calcium, samples were examined without contrast before staining by uranyl acetate and lead citrate. After treatment with oxalate–pyroantimonate, intracellular calcium is visible in TEM micrographs as electron-dense precipitates. As a control experiment, chelation of intracellular calcium with ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was tested on ultrathin sections (Ravindranath et al., 1994; Rozinek et al., 2006). Some unstained sections that showed calcium precipitates were treated for 10 min with an aqueous solution containing 10 mM EGTA which was dissolved using KOH and then examined under an electron microscope. The different developmental stages of spermatogenesis in the sterlet were recognized according to Mojazi Amiri & Takahashi (2006).

Unstained micrographs were used for intracellular calcium quantification. The area covered by calcium was

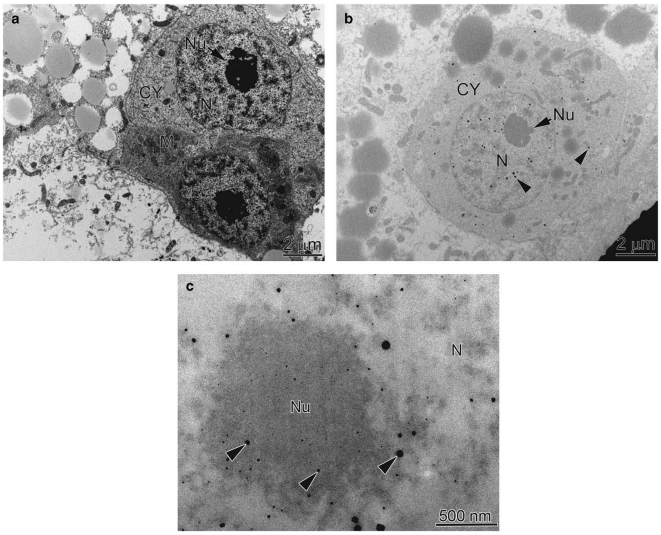

Figure 1. Transmission electron micrographs of the spermatogonium in sterlet *Acipenser ruthenus* testis. **a:** Micrograph stained with uranyl acetate and lead citrate shows two spermatogonia; (**b**) micrograph unstained with uranyl acetate and lead citrate showing calcium deposits in the nucleus (N) and cytoplasm (CY) (black arrowheads); (**c**) higher magnification of the nucleolus (Nu) exhibits numerous calcium deposits (black arrowheads). M, mitochondria.

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quantified using CellSens Dimension v.1.9 image software (Olympus) in the spermatogonia, spermatocyte, spermatid, and spermatozoon. In total, 36 cells in each stage from at least six fish were used for quantification. The level of calcium was assessed as the percent within the whole area of the respective cellular developmental stage. To normalize the data, proportional data were converted by angular transformation ($\arcsin\sqrt{p}$) before analysis by analysis of variance (ANOVA). Transformed data were checked for normality and homogeneity of dispersion using Kolmogrov–Sminov and Levene's tests, respectively. To compare the extent of calcium at different developmental stages, ANOVA was conducted, and Tukey's honest significant difference test was used to identify differences among groups at $p < 0.05$. Data in the text are presented as mean ± SEM of the untransformed data, but statistical tests were performed using transformed data.

Results

Spermatogonial ultrastructural was characterized by a large spherical and homogenous nucleus, as well as poorly condensed chromatin (Fig. 1a). Calcium deposits were observed in the nucleus and cytoplasm of spermatogonia (Fig. 1b). The nucleolus of spermatogonium also contained numerous smaller calcium deposits (Fig. 1c).

The ultrastructure of spermatocytes was characterized by a round nucleus with evenly dispersed chromatin and synaptonemal complexes (Fig. 2a). Calcium deposits were observed in the nucleus and cytoplasm of the spermatocyte (Figs. 2b, 2c).

Ultrastructural features of spermatids were characterized by a round nucleus with granular chromatin and considerable cytoplasm, and round or elliptical shaped mitochondria with an electron-dense matrix (Figs. 3a, 3b). In the mid-stage of spermatids, the nucleus undergoes an anterio-posterior elongation with an acrosome developing at the anterior part of the nucleus (Fig. 3b). The flagellum appeared in the posterior part of the cell (Fig. 3c). The spermatid contained calcium in the nucleus, acrosomal vesicle, and cytoplasm (Fig. 3d). Calcium deposits were transformed into an unbound form that covered a greater area in the cell (Fig. 3d).

The general structure of the sturgeon spermatozoon was characterized by an elongated nucleus, an acrosomal cap in the anterior part of the cell, a cylindrical midpiece, and a flagellum (Figs. 4a, 4b). Large amounts of unbound calcium were detectable in the nucleus while relatively lower amounts were observed in the acrosome (Figs. 4c, 4d). Sporadic calcium deposits were detected in the midpiece and flagellum (Fig. 4e). The intracellular calcium in the nucleus was mostly present as an unbound form in the late stages of spermatogenesis (Figs. 4c, 4d).

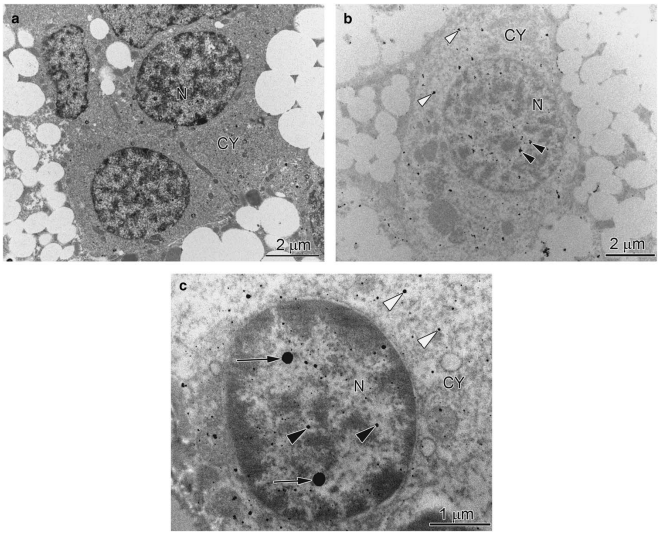

Figure 2. Transmission electron micrographs of the spermatocyte in sterlet *Acipenser ruthenus* testis. **a:** Micrograph stained with uranyl acetate and lead citrate showing spermatocytes with round nuclei; (**b**) micrograph unstained with uranyl acetate and lead citrate showing calcium deposits within the nucleus (N) (black arrowheads), cytoplasm (CY) (white arrowhead); (**c**) higher magnification of unstained spermatocyte with large (arrows) and small (arrowheads) calcium deposits inside the N, the calcium deposits also are clearly visible in the CY (white arrowheads).

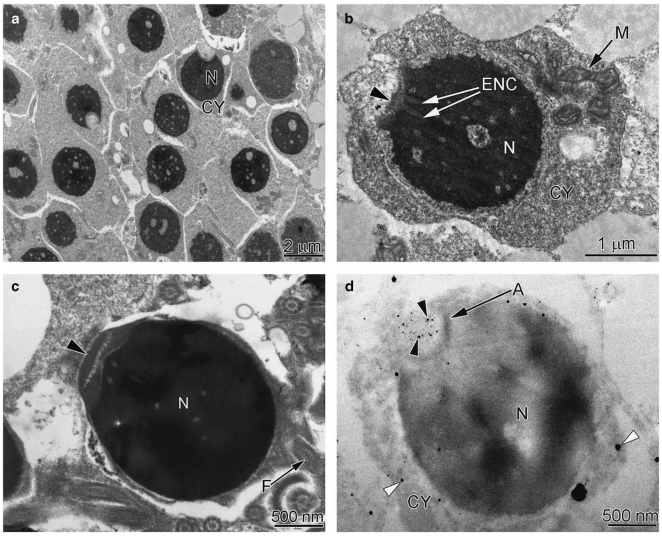

Figure 3. Transmission electron micrographs of the spermatid in sterlet *Acipenser ruthenus* testis. **a:** Micrograph stained with uranyl acetate and lead citrate shows a population of spermatids in different stages of development; (**b**) micrograph of round spermatid stained with uranyl acetate and lead citrate exhibits a developing acrosomal vesicle (black arrowhead) on the anterior part of the nucleus (N) with related primitive endonuclear canals (ENC) (white arrows); (**c**) micrograph of elongating spermatid stained with uranyl acetate and lead citrate showing a developing acrosomal vesicle (black arrowhead); (**d**) micrograph of an elongating spermatid unstained with uranyl acetate and lead citrate showing calcium within the N, acrosomal vesicle (black arrowheads) and cytoplasm (CY) (white arrowheads). Note that calcium in the N is mostly present as an unbound form. A, acrosome; F, flagellum; M, mitochondria.

Treatment with EGTA in controls showed specificity of the reaction, with spaces in the micrographs indicating intracellular calcium chelated by EGTA (Figs. 5a, 5b).

Quantification of Calcium Deposits

No significant difference ($p > 0.05$) was found in quantities of calcium between spermatogonium ($8.3 \pm 3.3\%$) and spermatocyte ($10.4 \pm 3.7\%$) stages. The proportions of area covered with calcium was increased significantly ($p < 0.05$) in the spermatid ($67.1 \pm 5.1\%$) and the spermatozoon ($74.8 \pm 3.0\%$) compared with spermatogonium and spermatocyte stages. There was no significant difference ($p > 0.05$) in the quantities of calcium between the spermatid and the spermatozoon stages.

Discussion

The present study illustrated changes in the distribution of intracellular calcium during sterlet spermatogenesis. Treviño et al. (1998) reported three types of ryanodine receptors (RyR), an important calcium pump, during spermatogenesis. However, they found RyR 1 and RyR 3 in spermatogenic cells and only RyR 3 in the mature spermatozoa. Activity of different types of calcium pumps during different stages of spermatogenesis might imply changes in the calcium balance and homeostasis during different developmental stages of male gamete production (Chiarella et al., 2004).

Evidence showed that calcium regulates important biological events in the spermatogonium (Ravindranath et al., 1994). Calcium signaling may have a role in regulation of the transition from a proliferating phase of spermatogonia to the last stages of spermatogenesis (Chiarella et al., 2004; Amemiya et al., 2007). The importance of calcium in the processes of cellular growth, differentiation and early stage of DNA synthesis for nuclear envelope breakdown has been proposed (Means & Rasmussen, 1988). It has been reported that depletion of intracellular calcium using some chemicals such as thapsigargin prevented cell growth and proliferation in spermatogonia (Treviño et al., 1998). Chiarella et al. (2004) stated that calcium signaling of all subtypes of RyRs likely contribute in the regulation of the spermatogonia transition from a proliferating phase to meiosis and also in advanced developmental phases.

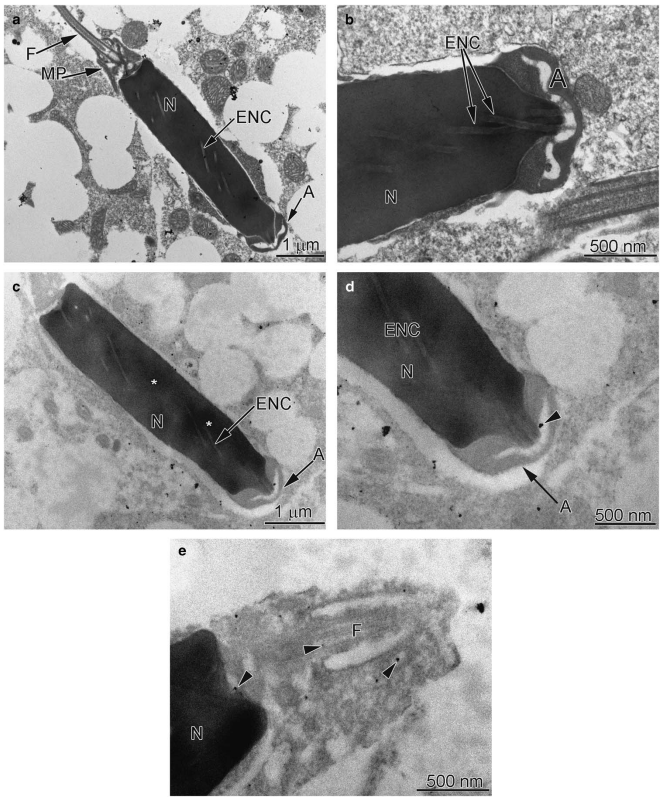

Figure 4. Transmission electron micrographs of the spermatozoon in sterlet *Acipenser ruthenus* testis. **a:** Longitudinal sagittal section of the spermatozoon stained with uranyl acetate and lead citrate showing different parts of the cell; (**b**) higher magnification longitudinal section stained with uranyl acetate and lead citrate exhibits acrosome (A) and endonuclear canals (ENC); (**c**) spermatozoon micrograph unstained with uranyl acetate and lead citrate showing distribution of a large amount of calcium as an electron-dense mass within nucleus (N) (white asterisks); (**d**) higher magnification micrograph of the spermatozoon unstained with uranyl acetate and lead citrate indicating calcium in the N and the A (arrowhead); (**e**) micrograph unstained with uranyl acetate and lead citrate showing sporadic calcium deposits (arrowheads) in the midpiece (MP) and flagellum (F). Note that calcium in the N is mostly present as an unbound form.

Calcium deposits were found in the rat spermatogonium and spermatocyte (Ravindranath et al., 1994). Interestingly, it has been proposed that spermatocytes possess the lowest concentration of calcium among the population of germ cells which gradually increases in subsequent stages of germ cell differentiation (Santi et al., 1998). This phenomenon has been supported by evidence in rat seminiferous tubules during spermatogenesis that shows significant elevation of calcium from spermatogonia to early spermatids (Hagiwara & Kawa, 1984).

We observed that calcium in the spermatogonium and spermatocyte stages were sequestered in deposit form which was scattered in limited areas (8–10%) of these cell types. It has been shown that calcium in Crane fly spermatocytes is not free, but rather is sequestered (Forer et al., 1980). This fact emphasizes that intracellular calcium homeostasis is important in maintaining spermatocyte function; otherwise any increase in the level of free calcium leads to apoptosis (Ghanayem & Chapin, 1990; Li et al., 1997; Lizama et al., 2007).

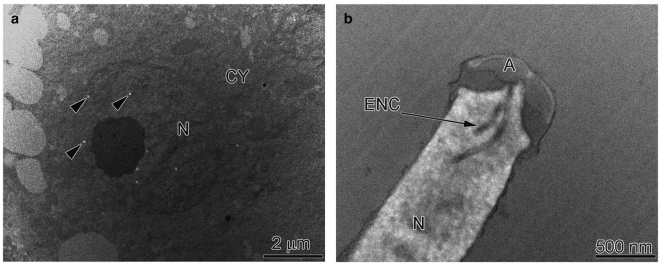

Figure 5. Control (**a**) spermatogonium showing electron lucent spaces (arrowheads) inside the nucleus (N) which represent chelation of calcium deposits after treatment with EGTA; (**b**) micrographs of a spermatozoon from sterlet *Acipenser ruthenus* testis after treatment with EGTA. The electron-lucent area within the N represents the calcium chelation with EGTA. Transmission electron micrographs are unstained. A, acrosome; CY, cytoplasm; ENC, endonuclear canals.

In the present study, the cytoplasm of spermatids exhibited calcium. According to Nakamura et al. (1993) the accumulation of calcium inside the cytoplasm has been attributed to "calciosomes," as specific calreticulin protein has been detected in both round spermatids and mature spermatozoon. Similar observations have been reported in the spermatid of rat testis (Ravindranath et al., 1994). In our study, the most interesting feature came from the sterlet spermatozoon where a relatively large area of the cell was covered by intracellular calcium. There is extensive evidence in such aquatic animals, including marine invertebrates, molluscs and echinoids that show the spermatozoon acrosome reaction is a calcium dependent event (Dan, 1956; Talbot et al., 1976; Nijima & Dan, 1985). In mammalian spermatozoa it has been demonstrated that both the plasma and the acrosomal membranes contain calcium pumps with the latter serving as a store of calcium during acrosome reaction (Spungin & Breitbart, 1996). On the other hand, it has been proposed that internal calcium stores might control many physiological functions such as the acrosomal reaction (Chiarella et al., 2004). This is also demonstrated in rat, hamster, mouse, and dog spermatozoa, suggesting that the acrosome is a source of internal calcium storage (Walensky & Snyder, 1995). The functional role of calcium in late phases of spermiogenesis is to participate in the microtubule machinery, particularly when they are obviously involved in motility (Boutinard Roueue-Rosier et al., 1993).

Conclusion

In conclusion, the present study demonstrated different distributional forms of calcium within the various cell types during spermatogenesis in Sterlet. Although calcium is sequestered in the form of deposits in limited areas of the spermatogonium and spermatocyte, it is present as an unbound form in larger areas of spermatids and spermatozoa, which probably reflects changes in its physiological function and homeostasis in the regulation of cellular processes during male gamete production.

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CHAPTER 3

SUBCELLULAR DISTRIBUTION OF CALCIUM DURING SPERMATOGENESIS OF ZEBRAFISH, *DANIO RERIO*

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RESEARCH ARTICLE

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Subcellular distribution of calcium during spermatogenesis of zebrafish, *Danio rerio*

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Abstract

Calcium plays a variety of vital regulatory functions in many physiological and biochemical events in the cell. The aim of this study was to describe the ultrastructural distribution of calcium during different developmental stages of spermatogenesis in a model organism, the zebrafish (*Danio rerio*), using a combined oxalate–pyroantimonate technique. Samples were treated by potassium oxalate and potassium pyroantimonate during two fixation stages and examined using transmission electron microscopy to detect electron dense intracellular calcium. The subcellular distribution of intracellular calcium was characterized in spermatogonium, spermatocyte, spermatid, and spermatozoon stages. The area which is covered by intracellular calcium in different stages was quantified and compared using software. Isolated calcium deposits were mainly detectable in the cytoplasm and the nucleus of the spermatogonium and spermatocyte. In the spermatid, calcium was partially localized in the cytoplasm as isolated deposits. However, most calcium was transformed from isolated deposits into an unbound pool (free calcium) within the nucleus of the spermatid and the spermatozoon. Interestingly, in the spermatozoon, calcium was mainly localized in a form of an unbound pool which was detectable as an electron-dense mass within the nucleus. Also, sporadic calcium deposits were scattered in the midpiece and flagellum. The proportional area which was covered by intracellular calcium increased significantly from early to late stages of spermatogenesis. The extent of the area which was covered by intracellular calcium in the spermatozoon was the highest compared to earlier stages. Calcium deposits were also observed in the somatic cells (Sertoli, myoid, Leydig) of zebrafish testis. The notable changes in the distribution of intracellular calcium of germ cells during different developmental stages of zebrafish spermatogenesis suggest its different homeostasis and physiological functions during the process of male gamete development.

KEYWORDS
electron microscopy, oxalate–pyroantimonate, quantification, testis, ultrastructural localization

1 | INTRODUCTION

Spermatogenesis is a unique developmental sequence in animals in which small numbers of diploid spermatogonial stem cells produce a large number of highly differentiated spermatozoa (Schulz et al., 2010). Ultrastructure of fish spermatogenesis has been studied in some species including golden grey mullet, *Liza aurata* (Bruslé, 1981), Japanese black porgy, *Acanthopagrus schlegeli* (Gwo & Gwo, 1993), Atlantic cod, *Gadus morhua* (Rebours & Ottesen, 2013), and longtooth grouper, *Epinephelus bruneus* (Kim et al., 2013). Studies on zebrafish spermatogenesis have suggested four major developmental stages which include spermatogonium, spermatocyte, spermatid, and spermatozoon (Leal et al., 2009; Rupik, Huszno, & Klag, 2011; Schulz et al., 2010). The successive development in male gamete cells during animal spermatogenesis is accompanied by morphological alteration, and is regulated by several chemical and molecular mechanisms (Cerdà, Bobe, Babin, Admon, & Lubzens, 2008; Yamashita, 1998).

Calcium has been proposed to play a major role in the regulation of key intracellular events. Cells strongly regulate the calcium signaling process based on its function during each developmental stage (Darszon,

Nishigaki, Beltran, & Treviño, 2011; Petr et al., 2001 and Sedmiakova et al., 2003). Calcium contributes to growth, proliferation and cell death in the spermatogonium and spermatocyte cells in several mammalian species (Ravindranath, Papadopoulos, Vornberger, Zitzmann, & Dym, 1994; Treviño et al., 1998). Moreover, several key functions in spermatozoa such as motility are controlled by calcium in mammalian and fish (Alavi & Cosson, 2006; Sakata et al., 2002). It has also been demonstrated that intracellular calcium deposits undergo a typical sequence of dynamic changes during the development of gametes which may contribute to its function and facilitate intracellular calcium homeostasis in rat and fish (Golpour, Pšenička, & Niksirat, 2016a, 2016b; Ravindranath et al., 1994). However, limited morphological studies are available about intracellular calcium involvement in the function of spermatogenic cells during spermatogenesis, and its intracellular locations in the different germ cell types have remained largely unknown (Ravindranath et al., 1994).

Basic information regarding distribution of the intracellular calcium storage sites and its possible functions during gamete development may provide better understanding of the process of zebrafish spermatogenesis as a model for studying reproduction of vertebrates. The aim of present study was to localize and quantify intracellular calcium during different stages of spermatogenesis in zebrafish *D. rerio* using a combined oxalate–pyroantimonate technique.

2 | MATERIALS AND METHODS

2.1 | Animal

The zebrafish *Danio rerio* (Hamilton, 1822) (Teleostei, Cyprinidae) has been selected as a model organism due to several advantages including small size, robustness, short life cycle and the fact that under laboratory conditions it can be induced to breed all year round (Ribas & Piferrer, 2013). Adult zebrafish (wild type of the AB – line) were obtained from the stock of the Department of Animal Science at University of Brno, Czech Republic and transported into the aquarium facilities of the Research Institute of Fish Culture and Hydrobiology. They were kept in aerated aquaria where the temperature was maintained at 26 ± 1°C and the photoperiod was set at 12:12 L: D. Fish were fed daily with commercial food (Tetra Werke, Melle, Germany). This study was conducted according to the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia, Faculty of Fisheries and Protection of Waters.

2.2 | Ultrastructural localization of the intracellular calcium stores

Transmission electron microscopy in combination with oxalate–pyroantimonate technique was used as a tool to trace any change in intracellular calcium stores relative to morphological changes in cell developmental stages (Niksirat & Kouba, 2016; Ravindranath et al., 1994). Six male zebrafish were anaesthetized in ice water (Maack & Segner, 2003), killed by severing their spinal cord, and the gonads were dissected. Samples were cut into small pieces and immediately immersed in the primary fixative containing 2% glutaraldehyde, 2%

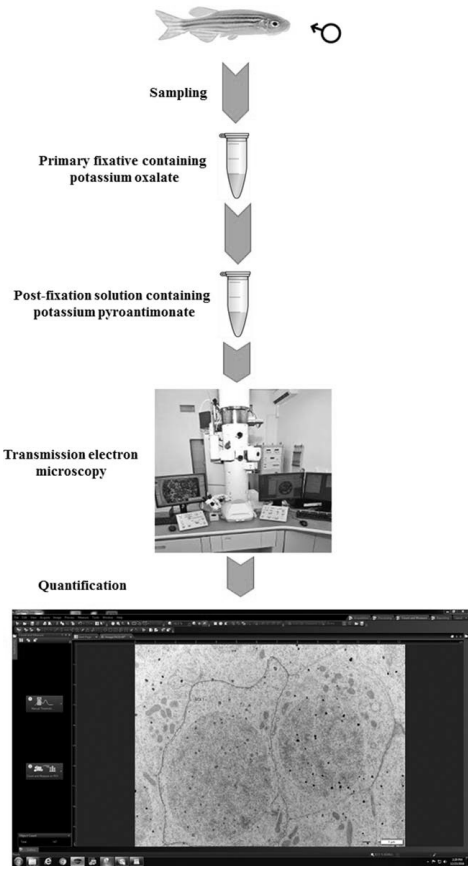

FIGURE 1 Schematic workflow of different stages of sample and data processing for localization and quantification of intracellular calcium in zebrafish *Danio rerio* spermatogenesis using oxalatae–pyroantimonate technique

formaldehyde, 90 mmol L^{-1} potassium oxalate, and 1.4% sucrose, pH adjusted to 7.4 using KOH, at room temperature and then transferred to a refrigerator at 4°C for 48 hr. The tissues were washed three times in distilled water, 15 min each, and post-fixed in 1% osmium tetroxide and 2% potassium pyroantimonate for 3 hr at 4°C. The excess pyroantimonate was removed through washing samples 3 times (15 min each time) in cold distilled water with pH raised to 10 using KOH (Golpour et al., 2016a; Ravindranath et al., 1994). Samples were dehydrated through an acetone series, and embedded in resin. A series of ultrathin sections were cut using a UCT Ultramicrotome (Leica Microsystems, Wetzlar, Germany) mounted on copper grids, double-stained with uranyl acetate and lead citrate, and examined with a 1010 transmission electron microscope (TEM) (JEOL Ltd., Tokyo, Japan) operating at 80 kV (Niksirat & Kouba, 2016). To facilitate detection of intracellular calcium

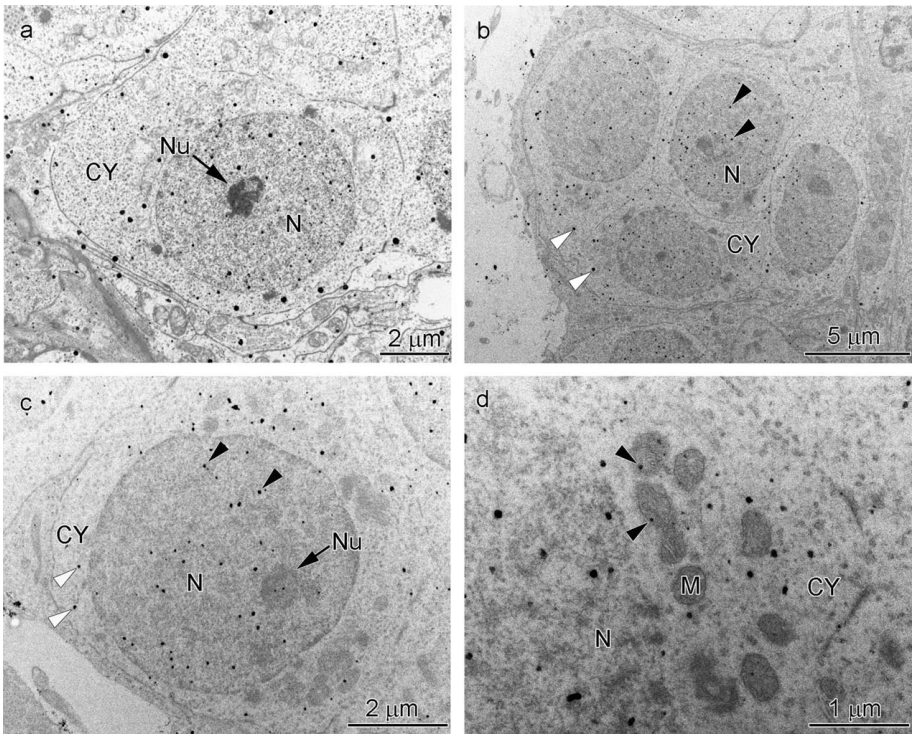

FIGURE 2 *Danio rerio*, transmission electron micrographs of the spermatogonium in the testis. (a) micrograph stained with uranyl acetate and lead citrate shows spermatogonium; (b) micrograph unstained with uranyl acetate and lead citrate showing isolated calcium deposits in the cytoplasm (white arrowheads) and nucleus (black arrowheads); (c) higher magnification of a single spermatogonium exhibits numerous isolated calcium deposits in the cytoplasm (white arrowheads) and nucleus (black arrowheads); (d) calcium deposits inside mitochondria. CY: cytoplasm, M: mitochondria, N: nucleus, Nu: nucleolus

in micrographs, samples were also examined before staining by uranyl acetate and lead citrate. After treatment with oxalate–pyroantimonate, intracellular calcium stores in the cell were visible in TEM micrographs as electron-dense precipitates. Both micrographs stained and unstained with uranyl acetate and lead citrate are presented in the results. As control, several unstained copper grids were randomly chosen and immersed in 10 mmol ethylene glycol tetraacetic acid (EGTA), which was dissolved in distilled water using KOH for 10 min to verify the specificity of the histochemical reaction (Ravindranath et al., 1994; Rozinek et al., 2006). The different developmental stages of spermatogenesis in zebrafish were determined according to Leal et al. (2009) and Rupik et al. (2011).

2.3 | Ultrastructural quantification of the intracellular calcium stores

Unstained micrographs were used for intracellular calcium quantification. The proportion of area which was covered by electron-dense calcium precipitates was quantified using CellSens Dimension v.1.9 image software (Olympus) in the four major developmental stages of spermatogenesis (Figure 1). Means from 12 cells in each stage were calculated. The level of calcium was assessed as the percent within the whole area of the respective cellular developmental stage. To normalize the data, proportional data were converted by angular transformation ($\arcsin\sqrt{p}$) prior to analysis by ANOVA. Transformed data were checked for normality and homogeneity of dispersion using Kolmogrov–Sminov and Levene's tests, respectively. To compare the extent of calcium at different developmental stages, ANOVA was conducted with subsequent Tukey's honest significant difference test to identify differences among groups at $p < .05$. Data are mean ± standard deviation (*SD*) of the untransformed data, but statistical tests were performed using transformed data.

3 | RESULTS

3.1 | Ultrastructural localization of the intracellular calcium stores

Spermatogonial ultrastructure was characterized by slightly oval or spherical shape with a single prominent nucleus and nucleolus (Figure 2a). The

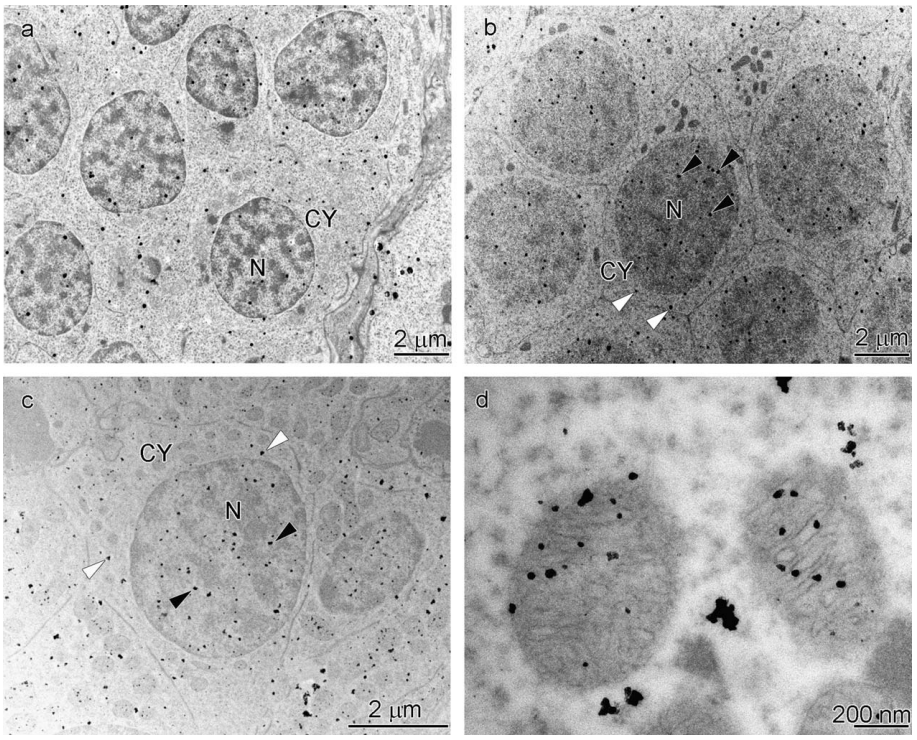

FIGURE 3 *Danio rerio*, transmission electron micrographs of the spermatocyte in the testis. (a) micrograph stained with uranyl acetate and lead citrate showing a group of spermatocytes; (b) micrograph unstained with uranyl acetate and lead citrate showing isolated calcium deposits within the cytoplasm (white arrowhead) and nucleus (black arrowheads); (c) higher magnification of a single spermatocyte unstained with uranyl acetate and lead citrate shows isolated calcium deposits in the cytoplasm (white arrowheads) and nucleus (black arrowheads); (d) calcium deposits inside mitochondria. CY: cytoplasm, N: nucleus

spermatogonium showed isolated calcium deposits which were scattered within the nucleus, cytoplasm, and mitochondria (Figure 2b–d).

The ultrastructure of a spermatocyte was defined by a large heterogenic nucleus and synaptonemal complexes (Figure 3a). In spermatocytes, isolated calcium deposits were detected within the nucleus, cytoplasm and mitochondria (Figure 3b–d).

Ultrastructure of a spermatid was characterized by a round nucleus, considerable cytoplasm, and condensation of heterochromatin masses (Figure 4a,b). The spermatid contained calcium in the cytoplasm and the nucleus (Figure 4b). A small part of intracellular calcium was still detectable in the form of the isolated deposits within the cytoplasm and mitochondria. However, isolated calcium deposits were transformed into a form of an unbound pool (free calcium) that covered a greater area in the nucleus (Figure 4c,d).

The general structure of the zebrafish spermatozoon was identified by a round nucleus which was entirely perpendicular to flagellar axis (Figure 5a). Isolated calcium deposits were sporadically detected in the midpiece and flagellum. Large amounts of unbound calcium were detected in the nucleus (Figure 5b). The intracellular calcium in the nucleus was mostly present as unbound pools in the late stages of spermatogenesis (Figures 4c and 5b).

Sertoli cells possess a triangular nucleus that is located in the border of the cysts (Figure 6a). Elongated myiod cells are located in the surrounding tubules of the testis (Figure 6b). Leydig cells with irregular or round nucleus are located in the wall of testis tubules (Figure 6c). Sertoli cells contained numerous calcium deposits in their nuclei. Smaller calcium deposits were also detected in the cytoplasm (Figure 6d). Large calcium deposits were observed in the nucleus of myiod cell (Figure 6e). Leydig cells also showed a large number of round calcium deposits in the nucleus and the cytoplasm (Figure 6f).

Holes and large electron-lucent spaces appeared in the cytoplasm and the nucleus of early and late stages of spermatogenic cells indicating EGTA-induced chelation of calcium in isolated deposits and unbound pools, respectively (Figure 7a–d). Intracellular distribution of calcium during different stages of the zebrafish spermatogenesis is summarized in Figure 8 using schematic illustrations.

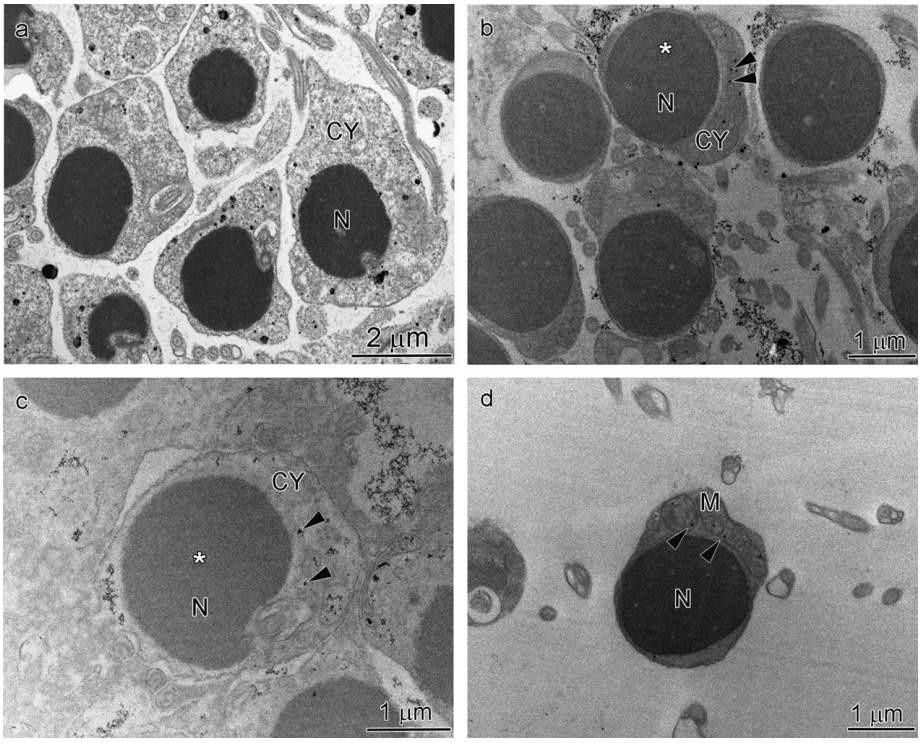

FIGURE 4 *Danio rerio*, transmission electron micrographs of the spermatid in the testis. (a) micrograph stained with uranyl acetate and lead citrate shows a population of spermatids; (b) micrograph of a group of spermatids unstained with uranyl acetate and lead citrate showing intracellular calcium in two forms of isolated deposits in the cytoplasm (arrowheads) and in the form of an unbound pool within the nucleus (white asterisk). (c) micrograph of a single spermatid unstained with uranyl acetate and lead citrate showing isolated calcium deposits within the cytoplasm (arrowheads) and unbound form of calcium in the nucleus (white asterisk); (d) calcium deposits inside mitochondria. CY: cytoplasm, M: mitochondria, N: nucleus

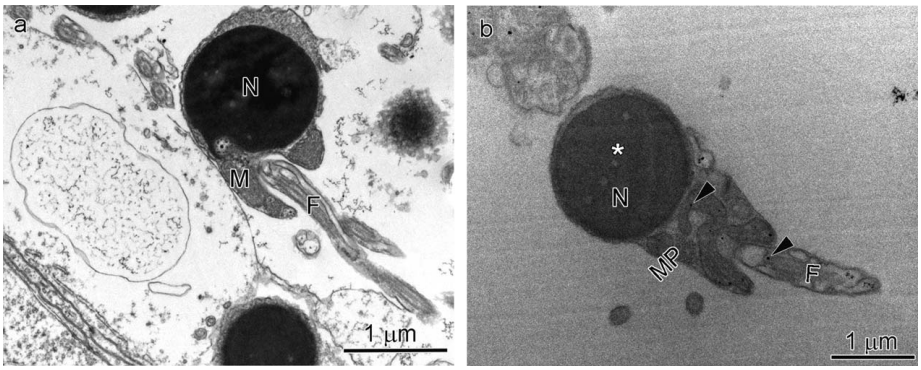

FIGURE 5 *Danio rerio*, transmission electron micrographs of the spermatozoon in the testis. (a) micrograph of the spermatozoon stained with uranyl acetate and lead citrate showing different parts of the cell; (b) micrograph unstained with uranyl acetate and lead citrate showing an unbound pool of calcium as an electron-dense mass within nucleus (white asterisk) and also sporadic isolated calcium deposits (arrowheads) in the midpiece and flagellum. F: flagellum, M: mitochondria, MP: midpiece, N: nucleus

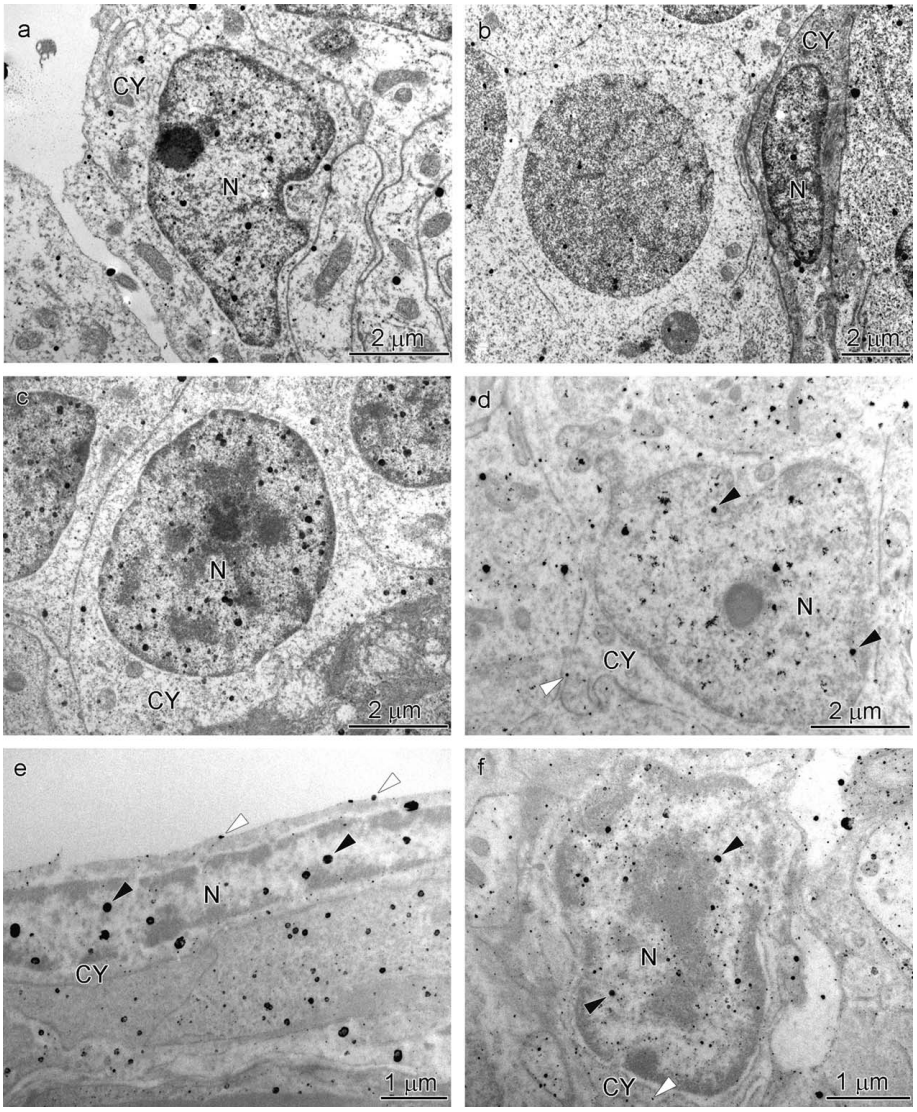

FIGURE 6 *Danio rerio*, transmission electron micrographs of the somatic cells in the testis. Micrographs stained with uranyl acetate and lead citrate showing (a) Sertoli, (b) myoid, and (c) Leydig cells; (d) micrograph unstained with uranyl acetate and lead citrate showing calcium deposits in the nucleus (black arrowheads) and the cytoplasm (white arrowhead) of a Sertoli cell, (e) micrograph unstained with uranyl acetate and lead citrate showing calcium deposits in nucleus (black arrowheads) and cytoplasm (white arrowheads) of a myoid cell, (f) micrograph unstained with uranyl acetate and lead citrate showing calcium deposits in nucleus (black arrowheads) and cytoplasm (white arrowhead) of a Leydig cell. CY: cytoplasm, N: nucleus

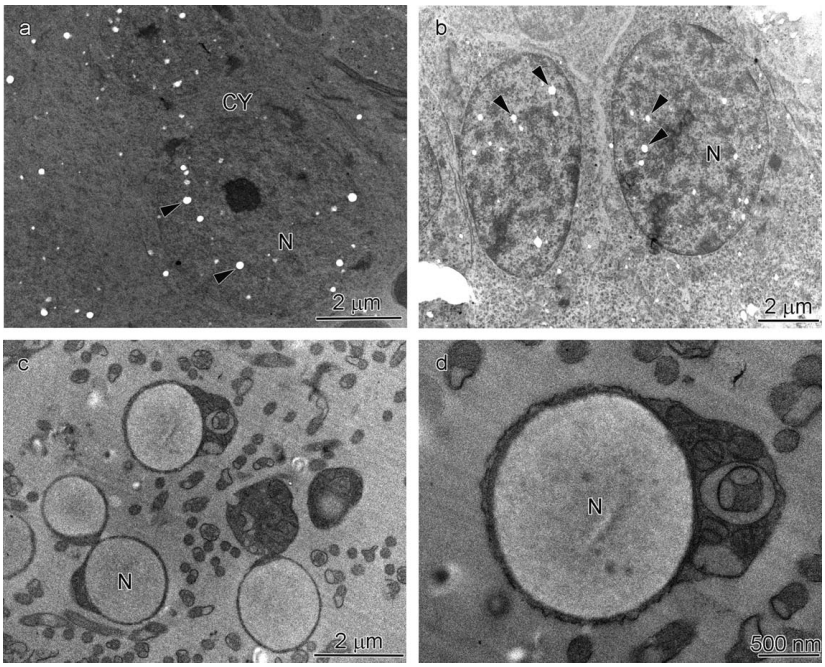

FIGURE 7 Controls. (a and b) arrowheads show holes inside the different parts of the cell which represents chelation of isolated calcium deposits after treatment with EGTA in spermatogonium and spermatocyte; (c and d) electron-lucent spaces in the nucleus of the spermatid and spermatozoon from zebrafish testis after treatment with EGTA. The spaces within the nucleus represents the chelation of unbound pools of calcium with EGTA. Transmission electron micrographs are unstained. CY: cytoplasm, N: nucleus

3.2 | Ultrastructural quantification of the intracellular calcium stores

The proportion of calcium deposits at different developmental stages of spermatogenesis is presented in Table 1. The proportional area which was covered by intracellular calcium increased significantly [F (3, 44) = 80.6, $p < .01$] from early to late stages of spermatogenesis. No significant difference was observed in quantities of intracellular calcium between spermatogonium and spermatocyte stages. The proportion of area which was covered by intracellular calcium increased significantly ($p < .05$) in the spermatid and the spermatozoon compared with spermatogonium and spermatocyte stages. There were also significant differences ($p < .05$) in the quantities of calcium between the spermatid and the spermatozoon stages. The highest area which was covered by intracellular calcium was detected in the spermatozoon.

4 | DISCUSSION

The present study showed that intracellular calcium is present in two distinctive forms during different stages of spermatogenesis in zebrafish. While most calcium is deposited in limited areas during the spermatogonium and spermatocyte stages, it is mostly detected as unbound pools (free calcium) which were released from dense isolated deposits of earlier stages of spermatogenesis and distributed in larger areas of the spermatid and the spermatozoon particularly nucleus. Similar changes in the ultrastructural distribution of calcium were observed during spermatogenesis of sterlet, *Acipencer ruthenus* (Golpour et al., 2016b).

Intracellular calcium can be elevated by calcium influx into a cell through the plasma membrane, or by a release of calcium from internal stores. For example, it has been observed that calcium influx increased from spermatogonium to early spermatid during rat spermatogenesis (Hagiwara & Kawa, 1984). Similarly, Santi, Darszon, and Hernández-Cruz (1996) observed that intracellular calcium levels increased gradually at advanced stages of mouse spermatogenesis.

Several studies have shown activity of a variety of different types of proteins which are associated with calcium regulation during stages of mammalian spermatogenesis (Darszon et al., 2011; Santi et al., 1996; Treviño et al., 1998) that reflect shifts in calcium balance and homeostasis during different developmental stages of gamete production.

Change in the form of intracellular calcium has also been observed during zebrafish oogenesis. Larger calcium deposits were transformed

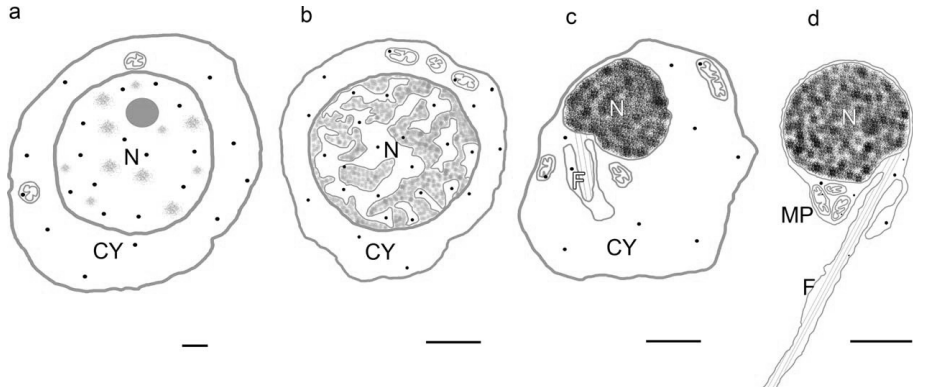

FIGURE 8 Schematic representation of the intracellular calcium distribution in different spermatogenic cells in zebrafish testis (for better contrast, the cell and intracellular calcium are illustrated by blue and black colors, respectively). In the spermatogonium (a) and spermatocyte (b) intracellular calcium was localized in the nucleus and cytoplasm as isolated deposits (black spots). The intracellular calcium in spermatid (c) was partially appeared as isolated deposits (black spots) in the cytoplasm but large amount of intracellular calcium was transformed into the form of an unbound pool within the nucleus (black region). In the spermatozoon (d), intracellular calcium was mainly localized in the form of an unbound pool within the nucleus (black region). However, some isolated calcium deposits are sparsely distributed in the midpiece and flagellum (black spots). CY: cytoplasm, F: flagellum, MP: midpiece, N: Nucleus. Scale bars: 1 μm

to smaller particles and finally into an unbound form during the development of cortical alveoli in the later stages of zebrafish oogenesis (Golpour et al., 2016a). Since cortical alveoli are considered as one of the sources of calcium release during egg activation (Schuel, 1978), transformation of calcium from isolated deposits into an unbound pool can facilitate its rapid release during egg activation (Golpour et al., 2016a).

Calcium regulates many vital functions during different stages of spermatogenesis such as growth, proliferation, and differentiation (Ravindranath et al., 1994; Treviño et al., 1998). However, a prolonged high level of intracellular free calcium is toxic and can cause negative effects on cell life including mitochondria damage, chromatin condensation, precipitation of phosphate and protein, activation of degradative enzymes such as proteases, nucleases, and phospholipases (Orrenius, McConkey, Bellomo, & Nicotera, 1989), and subsequent apoptosis (Ghanayem & Chapin, 1990; Li et al., 1997; Lizama, Alfaro, Reyes, & Moreno, 2007). Calcium is an especially sensitive indicator, and cells must maintain a suitable level all the times, because any fluctuation will severely impair cell function and subsequently kill it. In other word, calcium can change from a signal of life to a signal of death (Berridge, Bootman, & Lipp, 1998; Kazmierczak, Kempe, & Kremer, 2013). Therefore, germ cells during different stages of spermatogenesis need to regulate calcium levels in a way to avoid its intervention with gamete development.

TABLE 1 Proportion of area covered by intracellular calcium at different developmental stages of spermatogenesis

Stage	Minimum	Maximum	Mean ± *SD*
Spermatogonium	.06	0.15	$0.14 \pm .07^{a}$
Spermatocyte	.08	0.72	0.34 ± 0.21^{a}
Spermatid	5.04	36.82	17.85 ± 9.70^{b}
Spermatozoon	12.06	49.56	37.00 ± 14.92^{c}

Within columns, values marked with different alphabetic letters indicates significant difference at $p < .05$ as DETERMINED by Tukey's test. Values are expressed as percentage.

Spermatogonium and spermatocyte, as two early stages of spermatogenesis, are rapidly dividing cells. However, they contain less condensed chromatin than that found in cells of later stages of spermatogenesis (Rathke, Baarends, Awe, & Renkawitz-Pohl, 2014). Because high levels of free calcium can induce chromatin condensation or apoptosis (Orrenius et al., 1989), a major part of the intracellular calcium in the present study was observed to have been deposited in limited areas of zebrafish spermatogenic cells so as to keep calcium homeostasis and avoid activation of those deleterious processes. Our findings, were similar to those of Forer, Gupta, & Hall (1980) who showed that calcium in crane fly spermatocytes is not free, but rather is sequestered in some subcellular regions. Ravindranath et al. (1994) reported calcium deposits in the spermatogonium and spermatocyte of rat testis.

In addition, chromatin becomes highly condensed in the spermatid and the spermatozoon that may help to generate a compact hydrodynamic shape, protect the parental genome from physical and chemical damage, and involvement of protamines in epigenetic regulations. Chromatin condensation is regulated by several factors, including some proteins (Rathke et al., 2014; Saperas, Ribes, Buesa, García-Hegart, & Chiva, 1993), as well as calcium (Orrenius et al., 1989). Calcium in the spermatid and the spermatozoon of zebrafish forms unbound pools in the nucleus that could contribute in the regulation of chromatin condensation and compaction in these stages.

Spermatozoa are the final product of the spermatogenesis that eventually meets an egg for fertilization. Calcium plays important roles in the processes of egg activation and fertilization. Upon fertilization in almost all studied species, an increase in calcium starts at the point of spermatozoan entry and moves across the egg in a global wave as a sign of egg activation (Coward, Bromage, Hibbitt, & Parrington, 2002; Santella, Lim, & Moccia, 2004; Stricker, 1999). It has been shown that there are some spermatozoan factors including calcium, phospholipase and some proteins that are injected into the egg by the spermatozoon during gamete fusion and suggested to be responsible for initiating egg activation by triggering intracellular calcium wave (Anifandis, Messini, Dafopoulos, Daponte, & Messinis, 2016; Coward et al., 2003; Coward et al., 2005; Evans & Kopf, 1998; Jaffe, 1983; Swann & Parrington, 1999). A calcium bomb hypothesis states that the spermatozoon introduces calcium into the oocyte during fertilization that triggers a wave of calcium-induced calcium release (Jaffe, 1983; Machaty, 2016). But, experiments showed injection of pure calcium into eggs do not induce an egg activation-related calcium wave (Swann & Ozil, 1994; Swann & Whitaker, 1986). However, the fact that a crude extract of spermatozoa is able to induce egg activation shows that a combination of several factors including calcium, phospholipase, and some proteins together are necessary for egg activation by induction of the calcium mobilization from the egg internal stores (Coward et al., 2002; Machaty, Bonk, Kühholzer, & Prather, 2000; Swann, 1990; Wu, He, & Fissore, 1997). Our results also revealed that a large portion of calcium in the form of an unbound pool is concentrated in the head of mature spermatozoon as the first part that contacts the egg during fertilization. In addition, calcium transformed from dense and isolated deposits in the early stages of spermatogenesis into an unbound pool in the matured spermatozoon that facilitates its injection into the egg as one of the factors necessary for egg activation.

Calcium deposits were also observed in the somatic cells (Sertoli, myoid, and Leydig) of zebrafish testis. Ravindranath et al. (1994) observed calcium deposits in the somatic cells of rat testis. They reported that the nuclei exhibited larger, whereas the mitochondria in these cell types contained numerous smaller deposits. It has been demonstrated that calcium is essential for Sertoli cell estradiol biosynthesis, Leydig cell steroidogenesis and endothelin action on myoid cell (Fillipini et al., 1993; Grasso & Reichert, 1989; Sullivan & Cooke, 1986). Calcium regulates mammalian testis somatic cells via FSH and LH hormones. Also, treatment of Sertoli cells with FSH results in an increase in intracellular calcium (Means, Dedman, Welsh, Marcum, & Brinkley, 1979).

In conclusion, two distinctive forms of intracellular calcium stores including isolated deposits in limited areas of spermatogonia and spermatocytes, and unbound pools in larger areas of spermatids and spermatozoa were detected during zebrafish spermatogenesis. Change in the subcellular distribution of intracellular calcium during spermatogenesis reflects regulation of calcium based on its homeostasis and specific functions during different stages of male gamete development.

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CHAPTER 4

SUBCELLULAR LOCALIZATION OF CALCIUM DEPOSITS DURING ZEBRAFISH (*DANIO RERIO*) OOGENESIS

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Subcellular localization of calcium deposits during zebrafish (*Danio rerio*) oogenesis

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ABSTRACT

Calcium plays prominent roles in regulating a broad range of physiological events in reproduction. The aim of this study was to describe the subcellular distribution of calcium deposits during stages of oogenesis in zebrafish using a combined oxalate–pyroantimonate technique. The oocyte development of zebrafish was categorized into four stages: primary growth, cortical-alveolus, vitellogenic, and maturation, based on morphological criteria. Calcium deposits in the primary growth stage were detected in the cytoplasm, mitochondria, nucleus, and follicular cells. At the cortical-alveolus stage, calcium particles were transported from follicular cells and deposited in the cortical alveoli. In the vitellogenic stage, some cortical alveoli were compacted and transformed from flocculent electron-lucent to electron-dense objects with the progression of the stage. Calcium deposits were transformed from larger to smaller particles, coinciding with compaction of cortical alveoli. In the maturation stage, calcium deposits in all oocyte compartments decreased, with the exception of those in mitochondria. The proportion of area covered by calcium deposits in the mitochondria and cortical alveoli of oocytes at different stages of development was significantly different ($p < 0.05$). The extent of calcium deposits in the cortical alveoli of mature oocytes was substantially lower than in earlier stages. Basic information about calcium distribution during zebrafish oogenesis may contribute to better understanding of its role in oogenesis.

1. Introduction

During oogenesis, the oocyte undergoes developmental changes including four principal stages of oocyte growth (Wallace and Selman, 1981, 1990; Tyler and Sumpter, 1996; Jalabert, 2005). The basic pattern of oogenesis and its regulation is similar in all teleosts (Tyler and Sumpter, 1996; Clelland and Peng, 2009). The morphological characteristics of oocytes provide information on the dynamics of oogenesis, including final maturation and ovulation (Andrade et al., 2001). The ultrastructure of ovarian tissue has been used to clarify the reproductive cycle of teleosts (Grier and Taylor, 1998; Lo Nostro et al., 2003), since it provides reliable and accurate insight into the reproductive status of the female. Ultrastructural aspects of oogenesis have been assessed in fish species including *Pagrus major* (Matsuyama et al., 1991), zebrafish, *Danio rerio* (Selman et al., 1993), *Oreochromis niloticus* (Nakamura et al., 1993), common snook *Centropomus undecimalis* (Grier, 2000), bluefin tuna *Thunnus thynnus* (Abascal and Medina, 2005), and seagrass goby *Zosterisessor ophiocephalus* (Giulianini and Ferrero, 2009).

Calcium plays a pivotal role in diverse biological processes including meiosis resumption (Homa, 1995; Tosti, 2006), cortical reaction (Baker and Whitaker, 1978; Sardet and Chang, 1985), and embryo cell division (Whitaker and Patel, 1990; Chang and Lu, 2000; Whitaker, 2008). Numerous studies have demonstrated differences in the distribution of intracellular calcium deposits in oocytes depending on their stage of meiotic maturation. These oocytes differ in the ability to deliver calcium from intracellular deposits to the cytoplasm (Petr et al., 1997, 1999; Sedmıkova et al., 2003; Rozinek et al., 2006).

The zebrafish *D. rerio* possesses numerous features that make it a valuable model organism for the study of vertebrate development. Due to its asynchronous development of follicles, it is considered a prime model for biological research and developmental studies. The asynchronous mode of ovarian development also provides an excellent opportunity to study oocyte developmental stages (Nusslein-Volhard and Dahm, 2002). Despite the important role of calcium in the growth and differentiation of germ cell types in the ovary, data on the distribution of intracellular calcium deposits at different stages of oocyte development in zebrafish is lacking. In

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addition, location of the intracellular calcium storage sites in different ovarian cell types is not known. The aim of this study was to describe and compare the distribution of intracellular calcium deposits at different stages of oogenesis in zebrafish.

2. Materials and methods

2.1. Animals

Adult zebrafish were purchased from a commercial pet store and kept in aerated, temperature-regulated, 20 L aquaria. They were fed twice daily with commercial food (Tetra Werke, Melle, Germany) and with frozen live food. The water temperature was maintained at 26 ± 1 °C and the photoperiod was set at 12:12 L:D.

2.2. Preparation for light microscopy and calcium localization

Six female zebrafish were anaesthetized in ice water and killed, and the gonads were dissected. Then ovaries were removed and immediately immersed in a fixative of 2% glutaraldehyde, 2% formaldehyde, 90 mM potassium oxalate, and 1.4% sucrose, pH 7.4, at 4 °C. The tissues were washed three times, 15 min each, in distilled water and post-fixed in 1% osmium tetroxide and 2% potassium pyroantimonate for 3 h at 4 °C. Samples were rinsed thoroughly 3 times for 15 min each in cold distilled water with pH raised to 10 using KOH (Ravindranath et al., 1994). Samples were dehydrated through an acetone series, and embedded in resin (EPON). A series of ultrathin sections were cut using a UCT Ultramicrotome (Leica Microsystems, Wetzlar, Germany) mounted on copper grids, double-stained with uranyl acetate and lead citrate, and examined with a 1010 transmission electron microscope (TEM) (JEOL Ltd., Tokyo, Japan) operating at 80 kV (Niksirat et al., 2015). For better detection of calcium deposits, samples were examined without contrast prior to staining by uranyl acetate and lead citrate. After treatment with oxalate–pyroantimonate, calcium deposits in the ovary are visible in TEM micrographs as electron-dense precipitates. As a control to determine the specificity of the histochemical reaction, several copper grids were randomly selected and immersed in 10 mM ethylene glycol tetraacetic acid (EGTA), for 10 min to chelate the calcium pyroantimonate precipitate from the ultrathin sections (Ravindranath et al., 1994; Rozinek et al., 2006). For light microscopy, sections (1.5–2.0 μm) were cut, stained with methylene blue, and examined by light microscope (Leica DM 750, USA).

2.3. Calcium deposit quantification and statistical analysis

The proportion of area covered by calcium deposits in each organelle at different developmental stages was quantified using CellSens Dimension v.1.9 image software (Olympus). The level of calcium deposits was assessed as the percent of the area within the respective organelle. The percent was calculated for nucleus, nucleoli, mitochondria, and cortical alveoli. Since it did not show normal distribution, proportional data were converted by angular transformation (arcsin $\sqrt{p}$) prior to analysis by ANOVA. Transformed data were checked for distribution characteristics and homogeneity of dispersion using Kolmogrov–Sminov and Levene's tests, respectively. To compare the extent of calcium deposits at different developmental stages, ANOVA was conducted, and Tukey's honest significant difference (HSD) test was used to identify differences among groups at $p < 0.05$. Data are mean ± SD of the untransformed data, but statistical tests were performed using transformed data.

3. Results

3.1. Stage 1: primary growth

The first stage was designated as Primary growth and divided into three phases. A typical characteristic of this stage is a large nucleus relative to the volume of cytoplasm, and peripherally located nucleoli. In the initial phase, several oocytes are clustered in a nest (Fig. 1a). In the second phase, oocytes became separated from the nest and developed a distinct follicular layer (Fig. 1b). Numerous calcium deposits were observed within the oocytes (Fig. 1c,d). Calcium deposits were particularly apparent in the cytoplasm and especially in the nucleus (Fig. 1e). In the third phase, the primary zona radiata (vitelline envelope) developed and contained calcium deposits (Fig. 1f).

3.2. Stage 2: cortical alveolus

Commencement of the cortical alveolus stage was indicated by the appearance of cortical alveoli within the oocyte (Fig. 2a,b). Calcium particles appeared to be transported from follicular cells into the cytoplasmic space via zona radiata pores to aggregated inside the cortical alveoli (Fig. 2c,d). This stage was characterized by continuous asymmetric enlargement of the germinal vesicle and proliferation of nucleoli. Calcium deposits were observed in the nucleus and cytoplasm of the oocyte (Fig. 2e).

3.3. Stage 3: vitellogenic stage

In the vitellogenic stage (Fig. 3a), the zona radiata progressively thickened. Most mitochondria were distributed in a belt between the zona radiata and inner organelles (Fig. 3b). Cortical alveoli were progressively distributed to the periphery of the oocyte, while the yolk bodies accumulated in the center of the cell (Fig 3a,b). Large amounts of calcium continued to be transported from follicular cells into the oocyte via the zona radiata and was accumulated inside the cortical alveoli (Fig. 3c). Cortical alveoli are compacted from flocculent electron-lucent to electron-dense bodies with the progression of this stage (Fig. 3d–f). Calcium deposits were transformed from larger to smaller particles coinciding with compaction of cortical alveoli (Fig. 3g,h).

3.4. Stage 4: maturation

During the maturation stage, the fence-like striations of the zona radiata completely disappeared and a simple architecture developed (Fig. 4a). The zona radiata acquired a wave-like form and decreased in thickness (Fig. 4b). The concentration of calcium deposits decreased in areas of follicular cells and oocytes (Fig. 4c,d). Calcium deposits were still visible in oocyte mitochondria (Fig. 4e). Treatment with EGTA in controls showed specificity of the reaction, with spaces in the micrographs indicating calcium deposits chelated by EGTA (Fig. 5a,b).

3.5. Quantification of calcium deposits

The proportion of intracellular calcium deposits in the organelles of oocytes at different developmental stages is presented in Table 1. There was a significantly greater ($p < 0.05$) area of calcium deposits in mitochondria in the cortical alveolus stage compared to the primary growth stage, and this remained consistent ($p > 0.05$) through the maturation stage. The area of calcium deposits in the cortical alveoli of the mature oocyte was significantly smaller ($p < 0.05$) than in earlier stages. There was no significant differ-

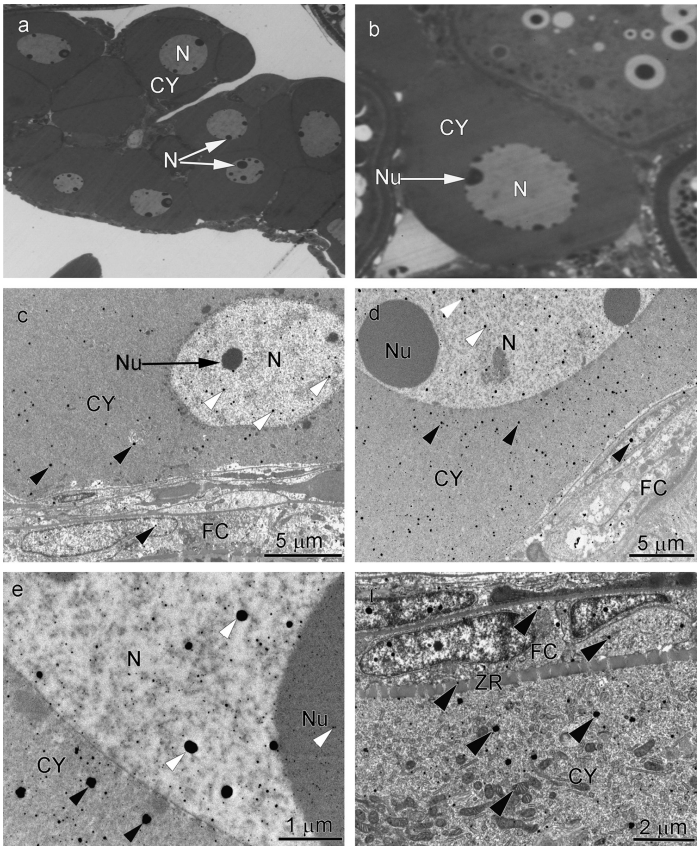

Fig. 1. Light and transmission electron micrographs of zebrafish ovary at three phases of primary growth. (a) Light micrograph of oocyte at first phase in the nest (magnification ×10), (b) oocyte separated from the nest with a follicular layer in the second phase (magnification ×60), (c) and (d) a distinct follicle is formed around oocyte, calcium deposits in the follicular cells, cytoplasm (black arrowheads), and nucleus (white arrowheads), (e) oocyte shows large calcium deposits inside cytoplasm (black arrowheads) and nucleus and smaller particles within nucleoli (white arrows), (f) primary zona radiata at the third phase, calcium deposits are visible inside follicular cells, zona radiata, cytoplasm, mitochondria (arrowheads). CY: cytoplasm, FC: follicular cells, N: nucleus, Nu: nucleoli, ZR: zona radiata (vitelline envelope). Transmission electron micrographs c–e were unstained.

Table 1
Proportion of area covered by calcium deposits in the organelles of egg at different developmental stages.

Organelles	Stage 1 (%)	Stage 2 (%)	Stage 3 (%)	Stage 4 (%)
Nucleus	0.47 ± 0.10^{a}	0.39 ± 0.19^{a}	–	–
Nucleolus	0.19 ± 0.12^{a}	0.23 ± 0.08^{a}	–	–
Mitochondria	0.32 ± 0.12^{a}	0.45 ± 0.15^{b}	0.48 ± 0.17^{b}	0.52 ± 0.09^{b}
Cortical alveoli	–	0.50 ± 0.09^{a}	0.53 ± 0.19^{a}	0.27 ± 0.12^{b}

Within rows, values marked with a similar letter did not differ significantly at $p < 0.05$ as determined by Tukey's test. Micrographs at different stages of egg development were obtained from 6 females analysed for quantification of calcium deposits using image analysis.
In the third and fourth stages, the nuclei and nucleoli become less evident and move to towards the exterior of the oocyte, respectively, and were omitted from quantification. Cortical alveoli begin to develop and appear at stage 2 of egg development; therefore there was no value for this organelle at stage 1.

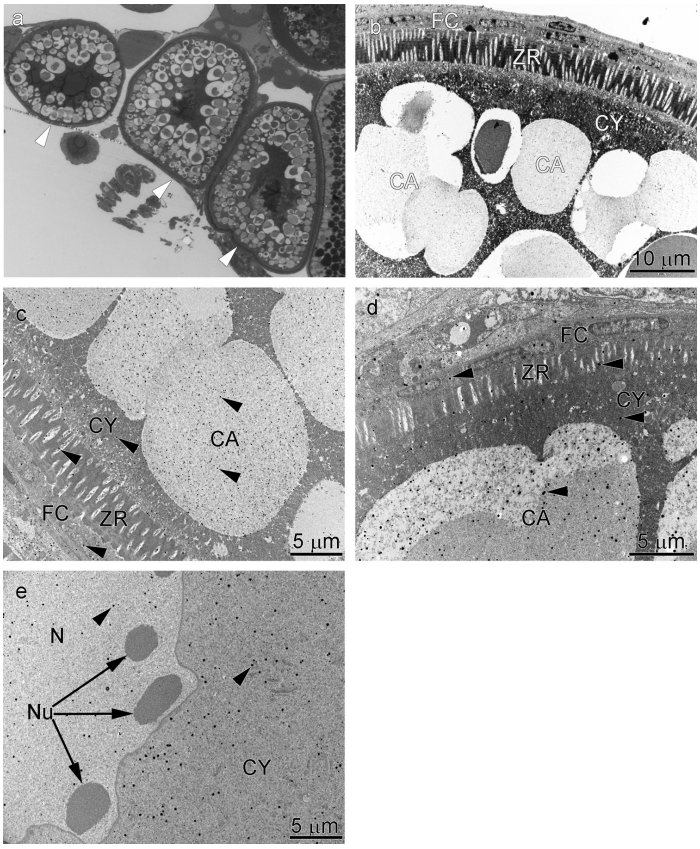

Fig. 2. Light and transmission electron micrographs of zebrafish ovary at cortical alveolus stage. (a) Light micrograph of oocyte at cortical alveolus stage (magnification ×10). The oocyte contains nascent cortical alveoli. (b) transmission electron micrograph of oocyte stained with uranyl acetate and lead citrate shows follicular cells surrounding the oocyte, distinct zona radiata, cytoplasm, and nascent cortical alveoli; (c) and (d) immature cortical alveoli containing numerous aggregated calcium deposits apparently transported from follicular cells into the cytoplasmic space via zona radiata pores, arrowheads show calcium deposits; (e) irregular enlargement of germinal vesicle and proliferation of nucleoli. Calcium deposits distributed in cytoplasm and nucleus of the oocyte (arrowheads). CA: cortical alveoli, CY: cytoplasm, FC: follicular cells, N: nucleus, Nu: nucleoli, ZR: zona radiata. Transmission electron micrographs c–e were unstained.

ence ($p > 0.05$) in the extent of calcium deposits in the nucleus and nucleoli at different stages.

4. Discussion

The study showed that calcium distribution changes during oogenesis in zebrafish, emphasizing the crucial role of calcium in oogenesis. Follicular cells deliver calcium to the cytoplasmic space via the zona radiata pores to aggregate inside organelles. The importance of granulosa cells in the follicular layer during oogenesis is further emphasized by the fact that these cells regulate a broad range of physiological events such as growth (Eppig et al., 1996), the resumption of meiosis (Tsafriri and Channing, 1975), and protein phosphorylation (Collona et al., 1989). The synchronous fluctuation of the level of calcium deposits in the cytoplasm of the oocyte and follicular cells is not surprising considering their proximity and wide variety of biologically active molecules that are regularly exchanged between them (Thibault et al., 1987; Rozinek et al., 2006). Shift in the liberation of intracellular calcium in oocyte is believed to be mediated through follicular cells and zona radiata at the oocyte surface, suggesting the existence of complex network of mutual exchange between the oocytes and granulosa cells during oogenesis (Thibault et al., 1987). Calcium has been identified in organelles of unfertilized sea urchin eggs by electron cytochemistry using the pyroantimonate technique (Gillot et al., 1990). Electron-dense deposits were observed on membranes of the cortical granule, mitochondria, and yolk vesicle. Deposits have also been observed within mitochondria and in the vitelline layer (Poenie et al., 1985; Sardet and Chang, 1985; Poenie and Epel, 1987; Gillot et al., 1989a). A study of sea urchin egg clearly showed the cortical granules to contain a higher level of calcium than did other large vesicular compartments, implying that the egg periphery contains more calcium than the interior (Gillot et al., 1989b). Studies have shown that the cortical layer of sea urchin eggs sequesters 14%

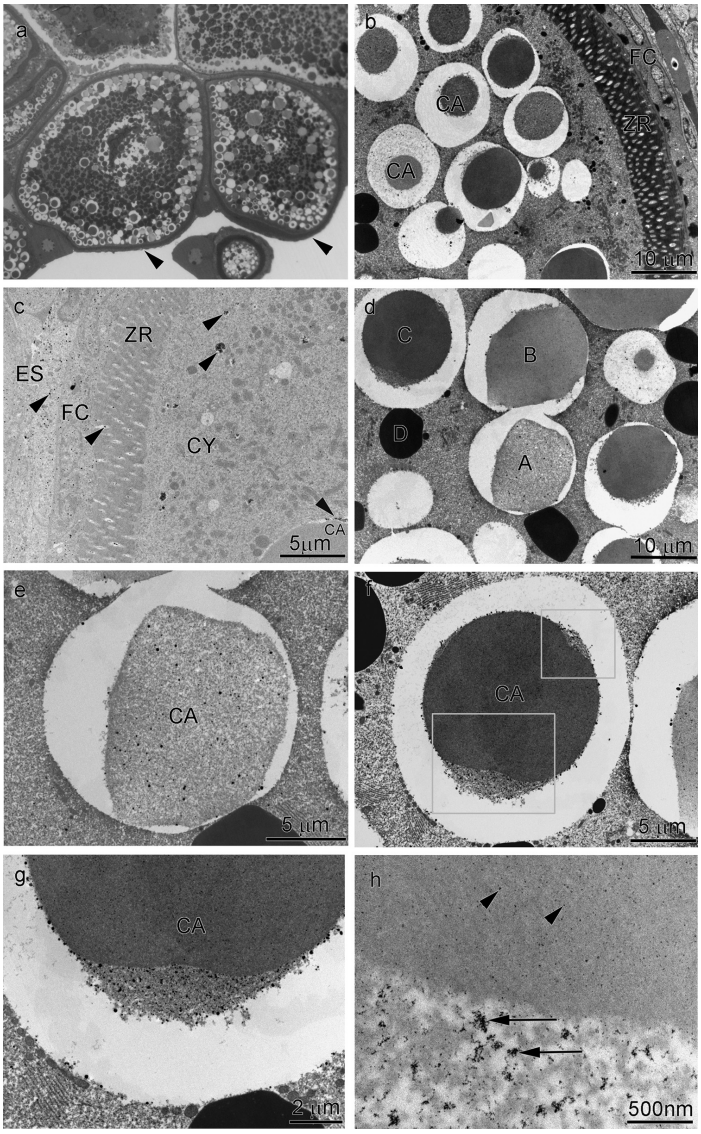

Fig. 3. Light and transmission electron micrographs of zebrafish ovary at vitellogenic stage. (a) Light micrograph of oocyte at vitellogenic stage (arrowheads; magnification ×10); (b) transmission electron micrograph of vitellogenic oocyte stained with uranyl acetate and lead citrate; (c) transmission electron micrograph shows transport of calcium deposits from extracellular space into cytoplasmic space via zona radiata pores (arrowheads); (d) A–D show stages of cortical alveoli compaction and transformation from flocculent electron-lucent to electron-dense bodies; (e) a flocculent electron-lucent body containing numerous calcium deposits; f: squares show compacting areas; (g) higher magnification micrograph of compacting area in a cortical alveoli; undefined (h) micrograph shows transformation of larger calcium deposits (arrows) to smaller particles (arrowheads) coinciding with the compaction of the cortical aleveoli. Note reduced size of calcium deposits within compacted area. CA: cortical alveoli, CY: cytoplasm, ES: extracellular space, FC: follicular cells, M: mitochondria, Y: yolk, ZR: zona radiata. Transmission electron micrographs c–h were unstained.

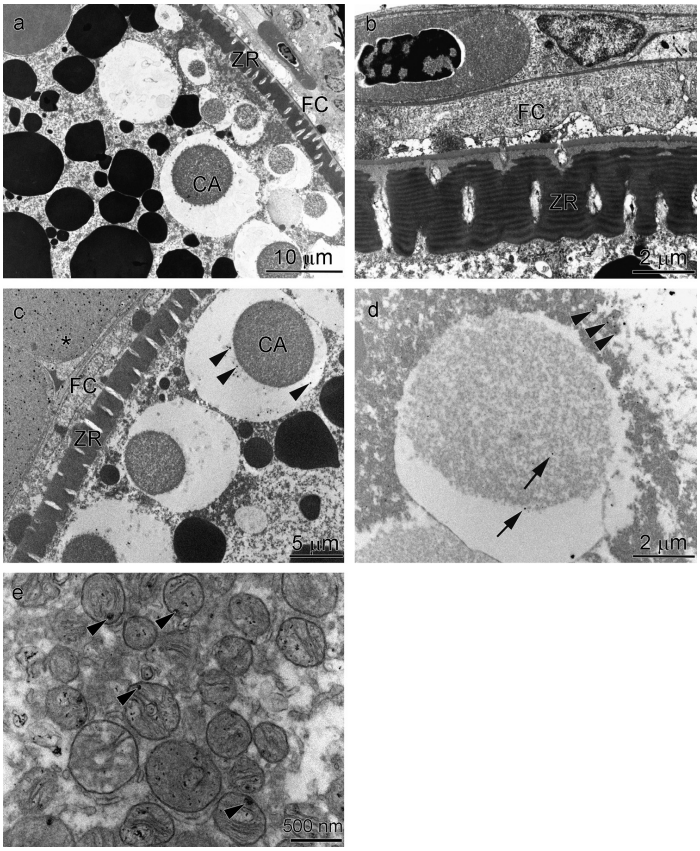

Fig. 4. Transmission electron micrographs of oocyte at maturation stage. (a) oocyte stained with uranyl acetate and lead citrate at maturation stage; (b) Higher magnification of zona radiata in maturation stage showing its wave-like form and reduced thickness; (c) small proportion of calcium deposits visible in the cortical alveoli (arrowheads), at upper left, star indicates a primary growth oocyte containing a large proportion of calcium deposits adjacent to a maturation stage oocyte; (d) higher magnification of a cortical alveoli with small proportion of calcium deposits (arrows), calcium deposits are more readily detected inside mitochondria (arrowheads), (e) high magnification micrograph shows calcium deposits inside mitochondria (arrowheads). CA: cortical alveoli, FC: follicular cells, M: mitochondria, ZR: zona radiata. Transmission electron micrographs c-e were unstained.

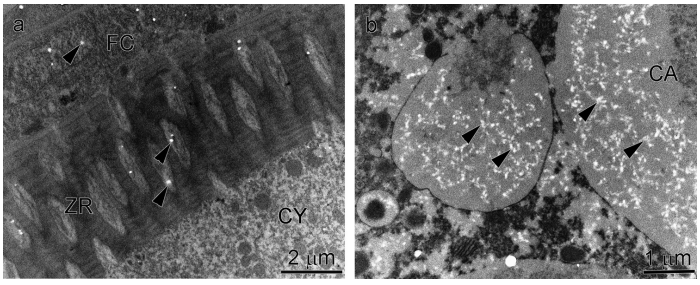

Fig. 5. Control. (a) and (b): transmission electron micrographs of oocytes show spaces previously occupied by calcium deposits (arrowheads) in the oocyte after sections were chelated with EGTA. CA: cortical alveoli, CY: cytoplasm, FC: follicular cells, ZR: zona radiata. Transmission electron micrographs were unstained.

of the total egg calcium (Gillot et al., 1991). Calcium stores in cortical granules are thought to stabilize the structure of the membrane or to possibly be the source of the intracellular calcium that is released at fertilization to initiate the cortical reaction and activate development (Schuel, 1978). The universal trigger of this activation process occurs with gamete fusion and a series of changes leading to cortical granule content discharge and embryogenesis (Miyazaki et al., 1993; Talmor-Cohen et al., 2002; Patel, 2004). The process is dependent on calcium stores in the organelles during oocyte maturation (Mehlmann et al., 1996). The mechanism of the activation process is poorly understood, but increase in cytoplasmic calcium levels appear to be involved (Hogben et al., 1998; Stricker, 1999; Machaty et al., 2000; Runft et al., 2002; Santella et al., 2004). It has also been reported that the content of the cortical alveoli participates in hardening of the vitelline envelope after ovulation, thereby preventing polyspermy (Cruz-Landim and Cruz-Hofling, 2000; Coward et al., 2002). A transient increase in cytoplasmic free calcium has been observed at egg activation in medaka (Ridgway et al., 1977), sea urchin (Steinhardt et al., 1977), frog (Wasserman et al., 1980), and mouse (Cuthbertson et al., 1981), indicating that the phenomenon is widespread. In addition, Ohta et al., (1990) stated that, at fertilization, cortical alveoli breakdown is accompanied by an explosive rise in intracellular free calcium ions derived from cytoplasmic stores. In zebrafish, it has been demonstrated that the duration of cytosolic calcium elevation resulting from the activation wave correlates with the time required to complete the cortical reaction (Hart and Yu, 1980; Lee et al., 1999). However, fertilization is assumed to be developed by calcium-stimulated calcium release, primarily from internal sources other than cortical alveoli. Major sources of participating calcium are the endoplasmic reticulum, cortical granules, mitochondria, nucleus, and yolk platelets (Schuel, 1978; Terasaki and Jaffe, 1991; Gilchrist et al., 1994; Kline, 2000; Sedmıkova et al., 2003). We have identified calcium deposits in the nucleus during zebrafish oogenesis. The presence of calcium-binding proteins that accumulate higher amounts of calcium in nucleus than in cytoplasm have been reported (Himpens et al., 1994; Gilchrist et al., 1994). Numerous factors, including state of cell differentiation and degree of protein phosphorylation, can lead to differing levels of calcium in the cytoplasm and nucleus of different cell types (Collona et al., 1989). Calcium deposits were also observed in the mitochondria, which can be explained by the large, internally negative, membrane potential across the inner membrane of the mitochondria resulting in intake of calcium across the inner membrane (Gunter and Gunter, 1994; Duchen, 2000; Gunter et al., 2004). Jouaville et al. (1995) demonstrated a noticeable role of calcium released from mitochondria in the modulation of the *Xenopus* oocyte. Calcium released from mitochondria of the mouse oocyte has been postulated to play a significant part in the regulation of meiosis resumption (Liu et al., 2001; Dumollard et al., 2004, 2006). Eisen and Reynolds (1985) reported no calcium liberation from the mitochondria of sea urchin *Arbacia punctulata* before egg fertilization, while a large calcium store was observed in the same organelle after fertilization. Significant reduction of calcium deposits in the studied organelles, except mitochondria, at the final stage of oocyte development might be due to the presence of calcium in an unbound form which is not detected by the oxalate–pyroantimonate technique. The unbinding of calcium in the mature oocyte may facilitate rapid transport of calcium during subsequent stages of egg activation, fertilization, and embryo development.

5. Conclusion

The present study has provided basic information on the presence of calcium in several organelles that likely serves as a source for calcium-dependent events in zebrafish oogenesis. The distribution of calcium deposits probably reflects the physiological status of the oocyte and may be involved in the regulation of cellular processes during oocyte maturation.

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CHAPTER 5

GENERAL DISCUSSION

Intracellular calcium distribution regulates many intracellular events (Ravindranath et al., 1994). The changes in the type of calcium distribution probably reflect the different physiological status of the germ cells and may cause a different mode of calcium signaling in the growth and differentiation of a variety of cell types during fish oogenesis. We found during early stages of gametogenesis that large amounts of intracellular calcium are sequestered as deposits to keep the level of free (unbound) calcium in the standard levels for cell. In addition, the distribution of calcium in an unbound pool (free) in the late stages of spermatogenesis (spermatid and spermatozoon) and oogenesis is probably due to facilitated release of calcium from related organelles (nucleus and cortical alveoli) as the main internal stores of calcium for rapid transport during egg and sperm fusion and egg activation. In parallel with these findings, some studies have demonstrated a differential distribution of calcium within the various cell types of the testis and ovary in mouse (Ravindranath et al., 1994; Sedmıkova et al., 2003).

In the first experiment, the distribution of intracellular calcium was assessed in sterlet A. ruthenus spermatogenesis as one of the key parameters to control many intracellular events such as growth and differentiation during different stages of gamete development. . In this study, ultrastructural distribution of intracellular calcium was evaluated during the different developmental stages of spermatogenesis in sterlet. Studies have confirmed that calcium signals are present not only in somatic cells but also in germ cells (Darszon et al., 1999; Stricker, 1999). In addition, it has further been assumed that intracellular calcium likely signals different specific cellular functions during spermatogenesis (Treviño et al., 1998; Chiarella et al., 2004). Presence of several calcium channels in spermatogenic cells and spermatozoa can probably explain their involvement in the control of calcium signals (Walensky and Snyder, 1995; Santi et al., 1998; Serranoa et al., 1999). In this study, two distinctive forms of intracellular calcium during different stages of spermatogenesis were illustrated. It is interesting to note that the calcium mostly appeared as isolated deposits in the early stages of spermatogenesis (the spermatogonium and spermatocyte) and were mainly localized in the nucleus and cytoplasm, while, in the spermatid and the spermatozoon, intracellular calcium transformed into an unbound form which was mainly localized in the nucleus.

In the second experiment, ultrastructural distribution of calcium during different developmental stages of spermatogenesis in a model organism, zebrafish (*Danio rerio*), was described using a combined oxalate-pyroantimonate technique. This study was performed to identify a pattern of distribution of calcium during spermatogenesis of teleost species for comparison with sturgeon species. Similar to our findings in sterlet, subcellular distribution of intracellular calcium was mostly characterized in a deposited form in the early stages of zebrafish spermatogenesis (spermatogonium and spermatocyte). Even, localization pattern of intracellular calcium in the spermatogonium and spermatocyte of zebrafish was similar to sterlet early stage cells, namely it was detectable in the cytoplasm and the nucleus. Presence of calcium as deposit (bound calcium) in the early stage of spermatogenesis might be due to keep homeostasis in maintaining spermatocyte function (Li et al., 1997; Lizama et al., 2007). Since, high levels of unbound intracellular calcium in the early stage of spermatogenesis can induce deleterious processes such as mitochondrial damage, chromatin condensation, precipitation of phosphate and activation of degradative enzymes proteases like nucleases and phospholipases (Orrenius et al., 1989) which subsequently lead to the impairment of cell function and apoptosis (Ghanayem and Chapin, 1990).

In advance stages of starlet and zebrafish spermatogenesis (spermatid and the spermatozoon), most calcium was transformed from isolated deposits into an unbound pool within the nucleus. This kind of calcium distribution (unbound) is likely due to its functional roles in late phases of spermiogenesis for participation in the microtubule machinery, particularly when they are obviously involved in motility (Boutinard Roueue-Rosier et al., 1993). This statement has been further supported by the proposition that explains that a large portion of calcium in the form of an unbound pool in the matured spermatozoon, facilitates its injection into the egg for induction of calcium wave which is observed during egg activation and fertilization (Jaffe, 1983; Coward et al., 2005; Anifandis et al., 2016; Machaty, 2016).

A third experiment was carried out to describe the subcellular distribution of intracellular calcium during different stages of oogenesis in a model organism, zebrafish (*D. rerio*) to better understand the calcium roles in the regulation of physiological events in reproduction. Distribution of calcium deposits was described and compared at different stages of oogenesis in zebrafish. Calcium deposits in the first stage of oocyte development (primary growth) were detected in the cytoplasm, mitochondria, nucleus, and follicular cells. At the second phase of oogenesis (cortical-alveolus), calcium particles were transported from follicular cells and deposited into the cortical alveoli. Calcium has been characterized in organelles of unfertilized sea urchin eggs by electron cytochemistry using the pyroantimonate technique (Gillot et al., 1990). On the other hand, calcium deposits were detected on membranes of the cortical granule, mitochondria, and yolk vesicle. A study of sea urchin eggs clearly showed the cortical granules contained a higher level of calcium than did other large vesicular compartments, implying that the egg periphery contains more calcium than the interior (Gillot et al., 1989).

The importance of calcium stores in cortical granules is further underlined by the fact that it's participation in stabilizing the structure of the membrane or possibly be the source of the intracellular calcium that is released at fertilization to initiate the cortical reaction and activate development (Schuel, 1978). This statement has been supported by evidence that shows at fertilization, cortical alveoli breakdown is accompanied by an explosive rise in intracellular free calcium ions derived from cytoplasmic stores (Ohta et al., 1990). The third phase of oocyte development in zebrafish is the vitellogenic stage which is accompanied by the compaction and transformation of flocculent electron-lucent cortical alveoli into electron-dense objects with the progression of the stage. In addition, larger calcium deposits were transformed to smaller particles and finally into an unbound form during the development of cortical alveoli in the later stages of zebrafish oogenesis. Since cortical alveoli are considered as one of the main sources of calcium release during egg activation (Schuel, 1978), transformation of calcium from isolated deposits into an unbound pool can facilitate its rapid release during egg activation.

In conclusion, the results of this study provide new and convergent insight into the regulation and functional roles of calcium during fish gametogenesis. During early stages of gametogenesis, most parts of intracellular calcium is sequestered in isolated deposits to keep the level of unbound calcium in suitable levels and avoid cell death or other negative effects that could be induced by high levels of free calcium. On the other hand, the distribution of calcium as unbound pool in the advanced stages of spermatogenesis (spermatid and spermatozoon) and late stages of oogenesis is probably due to facilitated release of calcium from related organelles (nucleus and cortical alveoli) as the main internal stores of calcium for rapid transport of calcium during egg and sperm fusion and egg activation.

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ENGLISH SUMMARY

Regulation of subcellular calcium during fish gametogenesis

Changes in the characteristics of spermatogenic and oogenic cells during gametogenesis may reflect a corresponding alteration in aspects of components such as calcium, which plays prominent roles in regulating a broad range of physiological events in animal reproduction. Basic information regarding distribution of intracellular calcium in different germ cells may provide better understanding of processes of reproduction in fish

The intracellular distribution of calcium during different developmental stages of spermatogenesis was studied in sterlet, *A. ruthenus*, using a combined oxalate–pyroantimonate technique. The distribution of calcium was described in spermatogonial, spermatocyte, spermatid, and spermatozoon stages. Calcium was localized as deposits mainly in the nucleus and cytoplasm of the spermatogonium and spermatocyte. The spermatid had calcium in the nucleus, developing acrosomal vesicle, and cytoplasm. Intracellular calcium transformed from scattered deposits in spermatogonia and spermatocyte stages into an unbound form in spermatid and the spermatozoon. Although calcium appeared in the form of deposits in limited areas of the early stage cells (spermatogonium and spermatocyte), it was present as an unbound form in larger areas of spermatids and spermatozoa especially the nucleus, which probably reflects changes in its physiological function and homeostasis of calcium during male gamete production.

Similar to sterlet sturgeon, ultrastructural distribution of calcium during different developmental stages of spermatogenesis was described in a model organism, zebrafish (*Danio rerio*), using a combined oxalate–pyroantimonate technique. The subcellular distribution of intracellular calcium was detected as deposits mainly in the cytoplasm and the nucleus of the spermatogonium and spermatocyte. Interestingly, large amounts of calcium were transformed from isolated deposits into an unbound pool (electrondense mass) within the nucleus of the spermatid and the spermatozoon. The alteration of intracellular calcium at different stages of *D. rerio* spermatogenesis can be related to specific function of each germ cell type during male gamete development. Unbound calcium in the nucleus of mature spermatozoon can be used for condensation of chromatin and induction of calcium wave during egg activation and fertilization.

Using a combined oxalate–pyroantimonate technique, the subcellular distribution of calcium deposits during stages of oogenesis in zebrafish was described. Calcium deposits were localized at different organelles within the egg during oocyte development. At the first stage of oocyte development (primary growth), calcium deposits were localized in the cytoplasm, mitochondria, nucleus, and follicular cells. At the cortical-alveolus stage, calcium particles were transported from follicular cells into the cortical alveoli. In the main stage of oocyte development (vitellogenic stage), some cortical alveoli were compacted and transformed from flocculent electron-lucent to electron-dense objects with the progression of the stage. Calcium deposits were transformed from larger to smaller particles, coinciding with compaction of cortical alveoli. On the basis of these data, we can propose that these changes (accumulation of calcium as unbound or free in the advanced stages) may be involved in the regulation of cellular processes such as the calcium wave during egg activation and fertilization.

In conclusion, the results of this study provides information regarding distribution and functional roles of calcium during fish gametogenesis. In addition, significant changes in the distribution of calcium during different developmental stages gametogenesis exhibit its different homeostasis and physiological functions during the process of gamete development. It is also noteworthy to note that, distribution of calcium as unbound pool (free) in the

advanced stages of spermatogenesis (spermatid and spermatozoon) and late stages of oogenesis is probably reflects regulation of calcium for its rapid transport during egg and sperm fusion as well as egg activation.

CZECH SUMMARY

Regulace subcelulárního vápníku v průběhu gametogeneze ryb

Změny ve vlastnostech samčích a samičích pohlavních buněk v průběhu gametogeneze mohou reflektovat změny v prvcích jako je vápník, který hraje významnou roli v regulaci mnoha fyziologických událostí při rozmnožování. Základní informace jako distribuce a lokalizace vápníku v různých typech zárodečných buněk mohou přinést lepší porozumění procesu reprodukce u ryb. Testikulární vývoj u generačních ryb jesetera malého (*Acipenser ruthenus*) byl studován v měsíčních intervalech prostřednictvím histologie a měření hladin sex hormonů. Výsledky identifikovaly čtyři různé fáze zahrnující klidové, předvýtěrové, výtěrové a povýtěrové období. Dále byly popsány změny gonadosomatického indexu testes. Hormonální profily 11-ketotestosteronu (11-KT) byly charakteristické vrcholem, který indikoval sezónní model vývoje gonád. Koncentrace 11-KT byly značně zvýšeny v průběhu spermatogeneze (před výtěrová fáze) a zůstaly stále vysoké i během před spermiačního období. Při reprodukční fázi došlo k markantnímu snížení 11-KT. Tato studie poskytuje představu o základních znalostech reprodukční biologie u samců jesetera malého a kompletně popisuje vývoj gonád, který nebyl popsán v přechozích studiích zaměřených na vývoj gonád jeseterů.

Intracelulární distribuce vápníku během různých vývojových fází spermatogeneze byla studována u jesetera malého pomocí kombinované oxalát-pyroantimonát techniky. Distribuce vápníku byla popsaná ve stadiích spermatogonie, spermatocytu, spermatidy a spermie. Vápník byl lokalizován jako depozity vyskytující se převážně v jádru a cytoplazmě spermatogonie a spermatocytu. U spermatidy byl vápník lokalizován v jádru, vyvíjejícím se akrosomálním váčku a cytoplazmě. Intracelulární vápník se transformoval z rozptýlených depozit u spermatogonie a spermatocytu do nevázané formy u spermatidy a spermie. Ačkoliv se vápník vyskytl ve formě depozitů v několika omezených oblastech u raných stadií buněk (spermatogonie a spermatocyt), je přítomný v nevázané formě ve větších oblastech u spermatid a spermií, především pak jádru, což pravděpodobně reflektuje jeho fyziologickou funkci a homeostázu během gametogeneze.

Podobně jako u jesetera malého byla popsána ultrastrukturální distribuce vápníku během vývojových stadií spermatogeneze u modelového organizmu dánia pruhovaného (*Danio rerio*), pomocí kombinované oxalát-pyroantimonát techniky. Subcelulární distribuce vnitrobuněčného vápníku byla detekována jako depozity nacházející se především cytoplazmě a jádru spermatogonie a spermatocytu. Zajímavostí je, že velké množství vápníku bylo transformováno z izolovaných depozitů do nevázané struktury (elektrondezní hmota) uvnitř jádra spermatidy a spermie. Úprava vnitrobuněčného vápníku během různých stadií spermatogeneze u dánia může být v souvislosti se specifickou funkcí různých typů zárodečných buněk během vývoje samčích gamet. Nevázaný vápník v jádru zralých spermií může být použit pro kondenzaci chromatinu a spuštění vápníkové vlny v průběhu aktivace vajíčka oplození.

Použitím kombinované oxalát-pyroantimonát techniky byla popsána subcelulární lokalizace depozit vápníku v průběhu oogeneze u dánia. Depozity vápníku byly lokalizovány uvnitř různých organel vajíček během vývoje oocytů. V prvním stadiu vývoje oocytů (primární růst), byly depozity vápníku lokalizovány v cytoplazmě, mitochondriích, jádru a folikulárních buňkách. V kortikálně alveolárním stadiu byly částice vápníku transportovány z folikulárních buněk do kortikálních alveol. V hlavním stadiu vývoje oocytů (vitelogenní stadium) byly některé kortikální alveoly stlačeny a přeměněny z vločkovitých elektron-Lucent na elektron-denzní objekty s progresí fázi. Depozity vápníku byly přeměněny z větších na menší částice, což se shoduje se stlačením kortikálních alveol. Na základě těchto výsledků můžeme navrhnout, že tyto změny (akumulace vápníku jako nevázaného nebo volného v pokročilých stadiích)

jsou součástí regulace buněčných procesů, jako je vápníková vlna v průběhu aktivace vajíčka a oplození.

Výsledky těchto studií poskytují nové nahlédnutí na informace o regulaci a funkční roli vápníku v průběhu gametogeneze u ryb. Během raných fází gametogeneze je většina vnitrobuněčného vápníku oddělena v izolovaných depozitech za účelem udržet hladinu nevázaného vápníku na vyhovujících hladinách a zároveň zabránit smrti buněk a dalším negativním vlivům, které mohou být způsobeny vysokým obsahem nevázaného vápníku. Na druhou stranu je distribuce vápníku ve volné formě v pokročilých fázích spermatogeneze (spermatidy a spermie) a pokročilých fázích oogeneze zapříčiněna uvolňováním vápníku z organel (jádro a kortikální alveoly) jakožto hlavních vnitřních zásobáren vápníku pro jeho rychlý transport v průběhu fúze gamet a aktivace vajíčka.

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LIST OF PUBLICATIONS

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Dadras, H., Sampels, S., **Golpour, A**., Dzyuba, V., Cosson, J., Dzyuba, B., 2017. Analysis of common carp *Cyprinus carpio* sperm motility and lipid composition using different in *vitro* temperatures. Anim. Reprod. Sci. 180, 37–43 (IF 2016 = 1.605)

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Golpour, A., Pšenička, M., 2015. Optimization of storage of sturgeon testicular cells in -80 °C. In: the 5th International Workshop on the Biology of Fish Gametes, 7–11 September, 2015. Ancona, Italy. (Poster presentation)

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Golpour, A., Pšenička, M., 2015. Optimization of storage of sturgeon testicular cells in -80 °C. In: the 5th International Workshop on the Biology of Fish Gametes, 7–11 September, 2015. Ancona, Italy. (Poster presentation)	2015

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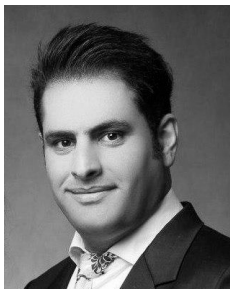

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