THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

ROLE OF ZINC IN THE MATURATION AND DEVELOPMENT OF MOUSE OOCYTES

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SPRING 2013

A thesis
submitted in partial fulfillment
of the requirements
for baccalaureate degrees
in Biology and Theatre
with honors in Biology

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ABSTRACT

Proper development and maturation of oocytes is very important in reproductive success. Many transition metals have recently been shown to have major roles in the development of oocytes. This study explores the role of intracellular zinc after fertilization, during meiosis II, and during meiosis I. Zinc appears to have a role during the metaphase-anaphase transition in both meiosis I and meiosis II. This study also explores the effects of zinc deficiency on maturation of oocytes and whether problems caused by zinc deficiency can be reversed. The difference in the presence of intracellular zinc in aged oocytes and young oocytes is also explored.

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Introduction

Quality of oocytes is an important factor in reproductive success. Interactions via paracrine signaling in the cumulus-oocyte complex (COC) is especially important for the control of oocyte and cumulus functions and development. Effects of oocytes on cumulus cells are clearly documented. Effects of oocytes on cumulus cells are mediated by GDF9 and BMP15 factors which activate various SMAD protein signaling pathways (Sugiera et al. 2007, Diaz et al. 2007). Cumulus cells are important for supplying the oocyte with nutrients (Eppig et al 2005, Sugiera et al. 2007, Su et al 2008) and are also responsible for inducing transcriptional silencing (De La Fuente and Eppig 2001) and preventing resumption of meiosis before ovulation (Pincus and Enzmann 1935, Zhang et al. 2010). It has also been shown that cumulus cells promote proper development of oocytes (Schroeder and Eppig 1984, Leibfried-Rutledge et al. 1989, Vanderhyden and Armstrong 1989, Chian et al. 1994, Zhang et al. 1995, Hashimoto et al, 1998, Eppig 2001, Su et al. 2004, Luciano et al. 2005, Wongsrikeao et al. 2005, Hussein et al. 2006, Johnson et al. 2008, Lee et al. 2011). The reciprocal interactions between cumulus cells and oocytes are thus necessary for oocyte development and consequent fertility.

Recently, it has been shown that transition metals, such as zinc, are important for meiotic progression. Zinc transporters are abundantly expressed on both cumulus cells and oocytes (Lisle et al. 2013). Increase in intracellular zinc of oocytes has been shown during meiotic maturation, which is the period between LH surge and ovulation (Kim et al. 2010). During this period the oocyte progresses from meiosis I and arrests at metaphase of meiosis II. Zinc has also been shown to be necessary for establishment of

metaphase II arrest (Suzuki et al. 2010b, Bernhardt et al. 2012). Oocyte activation and exit from meiosis II has been shown to require a lowering of cellular zinc to allow exit from meiosis II (Kim et al. 2011). Cumulus cells in particular have recently been shown to help mediate zinc homeostasis within the oocyte by preventing increase of free intracellular zinc in immature cells (Lisle et al. 2013). It has also been shown that zinc is required for chromatin methylation through a mechanism involving the generation of the methyl donor S-adenosylmethionine (SAM) (Tian and Diaz 2013). Supplementation with SAM has been shown to restore chromatin methylation in zinc deficient oocytes but effects on oocyte maturation are unknown.

Lack of zinc has also been shown to have consequences on oocyte maturation. Insufficient accumulation of intracellular zinc has been shown to lead to premature arrest of oocytes at telophase I (Kim et al. 2011). Zinc deficiency has been shown to cause defects, such as spindle defects, that have effects on oocyte maturation. (Tian and Diaz, 2012). Thus, zinc plays an important role in development and maturation of oocytes.

This study focuses on the changes in distribution of free intracellular zinc during maturation and the effect of SAM supplementation on oocyte maturation. The presence of free intracellular zinc will be explored during meiosis I and meiosis II. The effects of zinc deficient diet on the maturation of oocytes will also be explored. Presence of zinc in aged oocytes will also be determined.

Materials and Methods

Animals

Female CD1 mice (*Mus musculus)* were obtained from the research colony of the investigators. Ovaries were collected for *in vitro* experiments from 21-day old mice 48 hours after injection of 5 IU pregnant mare serum gonadotropin (PMSG) (National Hormone and Peptide Program, National Institute of Diabetes and Digestive Kidney Diseases, Torrance, CA). All animal use was reviewed and approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University.

Presence of zinc during MII after fertilization

COCs were collected from PMSG-primed mice (48 hours). Ovaries were collected and placed in a collection dish containing medium (bicarbonate buffered MEM-α (Life Technologies, Inc., Grand Island NY) with 5% Fetal Bovine Serum (FBS)). Cumulus-oocyte complexes (COC) were released from ovaries by puncture with syringe and needle and immediately separated into 4 groups and placed in a 4-well collection dish containing media (bicarbonate buffered MEM-α with 5% FBS and EGF). *In vitro* fertilization was performed after incubation overnight. Oocytes were stained with fluozin-3 AM indicator dye (2μM, Invitrogen, excitation 494/emission 516) for 15 minutes. Fluozin-3 binds to free or loosely boundh intraceullar zinc, but does not detect zinc that is tightly bound to proteins. Oocytes were then washed in fresh medium and imaged using an Axioscope fluorescence microscope (excitation 494/emission 516).

Oocytes were imaged after 1.5 hours after fertilization, 2 hours after fertilization and 3 hours after fertilization.

Rescue of zinc-deficient oocytes using SAM

COC were collected from PMSG-primed mice (48 hours) fed either a control diet or a zinc-deficient diet for 5 days prior to ovary collection. Diets were purchased from MP biochemical (Solon, OH) and were based on diet AIN76 with added zinc (control diet 29 mg Zn/kg; deficient <1mg Zn/kg) as described previously (Tian and Diaz 2013). Ovaries were collected and placed in a collection dish containing medium (bicarbonate buffered MEM-α (Life Technologies, Inc., Grand Island, NY) with 3 mg/ml BSA (Cohn Analog purified, Sigma) and EGF). COC were released from ovaries by puncture with syringe and needle and washed in fresh medium. COC were separated into 4 groups: control, zinc-deficient, SAM control and SAM zinc-deficient. SAM groups were placed in media containing MEM-α, 3mg/ml BSA, EGF and 1µl/ml S-(5'-adenosyl)-L-methionine (SAM) (100mM, Sigma). All groups were incubated overnight. After incubation oocytes were denuded from cumulus cells and fixed in PFA (4%) for 30 minutes. Oocytes were washed in PBST and BSA and stained with phalloidin. After staining, oocytes were washed and placed on glass slides with etched rings with Prolong Antifade with DAPI. Oocytes were imaged using an Axioscope 2 fluorescence microscope.

Presence of zinc at MI phase

Ovaries were collected from PMSG-primed mice (48 hours) and placed in medium (bicarbonate buffered MEM-α and BSA). COC were released from ovaries by

puncture with syringe and needle and washed in fresh medium. After washing COCs were divided into 2 groups: control and [N,N,N',N'-tetrakis(2-pyridylmethyl_ethylenediamin] (TPEN). TPEN is a zinc chelating agent that has been shown to disrupt oocyte maturation (Kim et al. 2010). Both groups were incubated for 8 hours in medium containing EGF. After incubation oocytes were loaded with fluozin-3 AM indicator dye and imaged using an Axioscope 2 fluorescence microscope. This experiment was repeated and the oocytes were incubated for 10 hours instead of 8 hours.

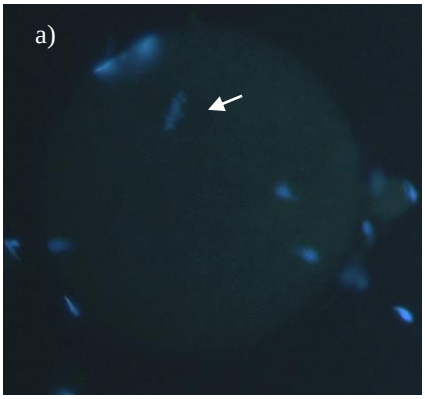
Comparison of intracellular zinc in oocytes of old and young mice

COC were collected from old mice (8-9 months) and young mice (3 weeks) induced to ovulate with PMSG and hCG. COC were released from oviducts of each group by puncture with syringe and needle. COC were washed in fresh medium and loaded with fluozin-3 AM indicator dye. Oocytes were imaged at the Microscopy and Cytometry Facility of the Huck Institutes of the Life Sciences using confocal microscopy.

Results

After fertilization zinc forms a bubble around DNA preparing to be separated in MII

After fertilization, oocytes were imaged to determine the presence of intracellular zinc. During all time periods after fertilization, oocytes presented with a distinct bubble outlined by zinc surrounding the meiotic spindle including the chromosomes at metaphase II (Figures 1a,b). This accumulation around the meiotic spindle suggests that zinc may be important for the lining up of the chromosomes along the meiotic spindle, or for attachment of microtubules to the centromere of homologous chromosomes in preparation for subsequent separation during anaphase II. Further research and observation would be necessary to determine the exact role of zinc during this period of time.



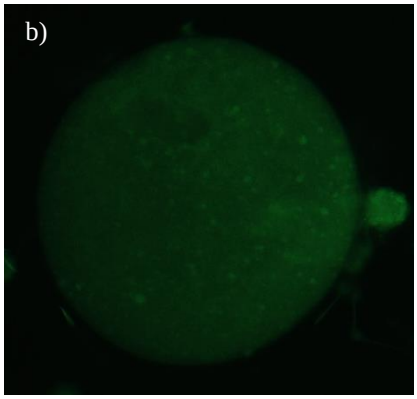


Figure 1 **2 hours after fertilization the chromosomes are aligned (arrow) (a). A bubble outlined by intracellular zinc surrounds the meiotic spindle (b).**

SAM did not appear to rescue zinc-deficient oocytes during maturation

After incubation in EGF with or without SAM, oocytes were imaged and determined to be in the germinal vesicle (GV) stage, meiosis I (M1) or meiosis II (M2) phases. The control group exhibited 77% of oocytes in the M2 phase while the zinc-deficient group exhibited 61% of oocytes in the M2 phase with a larger percentage of oocytes in the GV and M1 phases as compared to the control group (Table 1)(Figure 2).

Table 1: Percentage of oocytes in germinal vesicle (GV) stage, meiosis I (M1) and meiosis II (M2).

Percentage of Total	GV	M1	M2
Control	13%	10%	77%
Zinc Deficient	22%	17%	61%
SAM Control	0%	7%	93%
SAM Zinc Deficient	16%	20%	64%

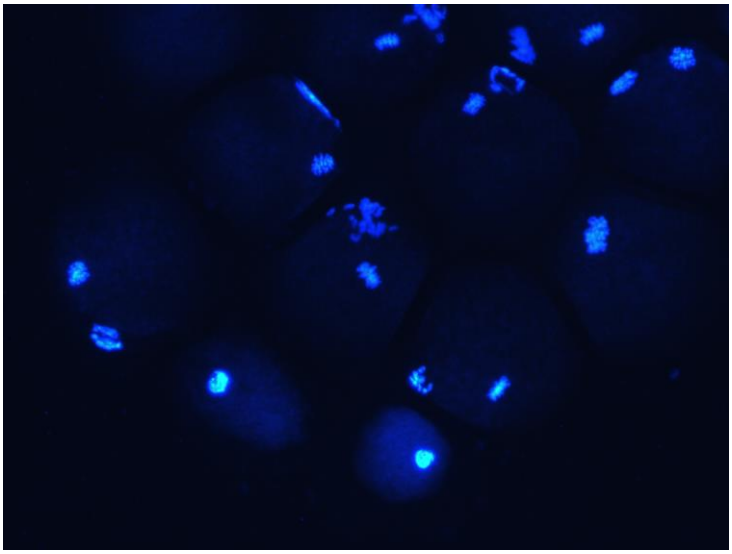


Figure 2 **An example of the different stages of oocytes after being treated with EGF. GV stage appears with the DNA in a circle. MI phase appears with the chromosomes aligned and MII phase appears with one set of chromosomes aligned and another set separated into what will become the polar body. This group came from the control group.**

The control SAM group exhibited a higher percentage (93%) of oocytes in the M2 phase than the regular control group and exhibited no oocytes in the GV stage. The zinc-deficient SAM group exhibited 64% of oocytes in the M2 phase which was very similar

to the percentage of oocytes in the M2 phase of the zinc-deficient group treated without SAM. This suggests that the addition of SAM did not affect the maturation of zinc-deficient oocytes. However, the control SAM group and the zinc-deficient SAM group had very different percentages of oocytes in the M2 phase while the percentage of oocytes in M2 between the control group and the zinc-deficient groups treated without SAM had more similar percentages. This suggests that SAM may have an effect on the maturation of oocytes that have freely available zinc.

At metaphase I zinc forms a bubble around the meiotic spindle

After 10 hours some of the control oocytes appeared to be in metaphase I while others appeared to be in anaphase I (Figures 3a, 3b, 3c, 3d). The chromosomes of the metaphase oocytes were not perfectly aligned but it was impossible to tell if the chromosomes was lining up or starting to separate. An outlining of zinc can clearly be seen surrounding the meiotic spindle, forming a bubble. This indicates that intracellular zinc is important during the metaphase I/anaphase I period, but it is uncertain whether it is important in lining up or separating the chromosomes. After 10 hours the oocytes treated with TPEN for 1 hour showed the chromosomes lined up with zinc surrounding the meiotic spindle (Figure 4a, 4b). Again, it was difficult to tell whether the chromosomes were lining up or beginning to separate. However, one of the oocytes appeared to show either a misalignment of the chromosomes or the beginning of an improper separation of the chromosomes to one side of the oocyte (Figures 4c, 4d). This may indicate that zinc is important for proper separation of the chromosomes during anaphase I, however, more research needs to be done to determine this.

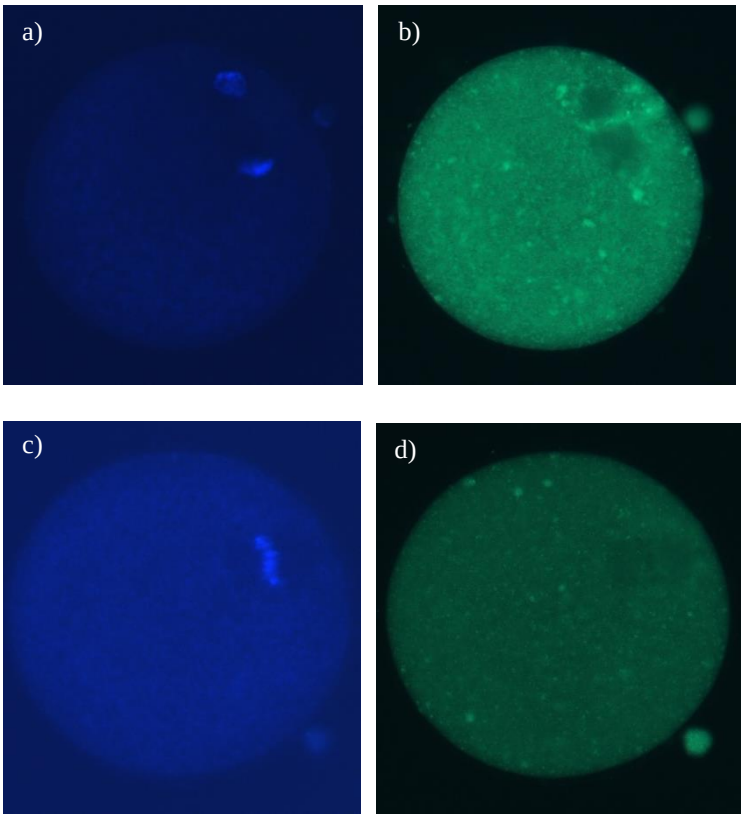


Figure 3 **After 10 hours control oocytes appeared to be in anaphase I, with the chromosomes separating (a). Zinc surrounds the sets of chromosomes forming two bubbles b). However, some oocytes appeared to still be in metaphase I (c) with one large bubble outlined with zinc surrounding the aligned chromosomes (d).**

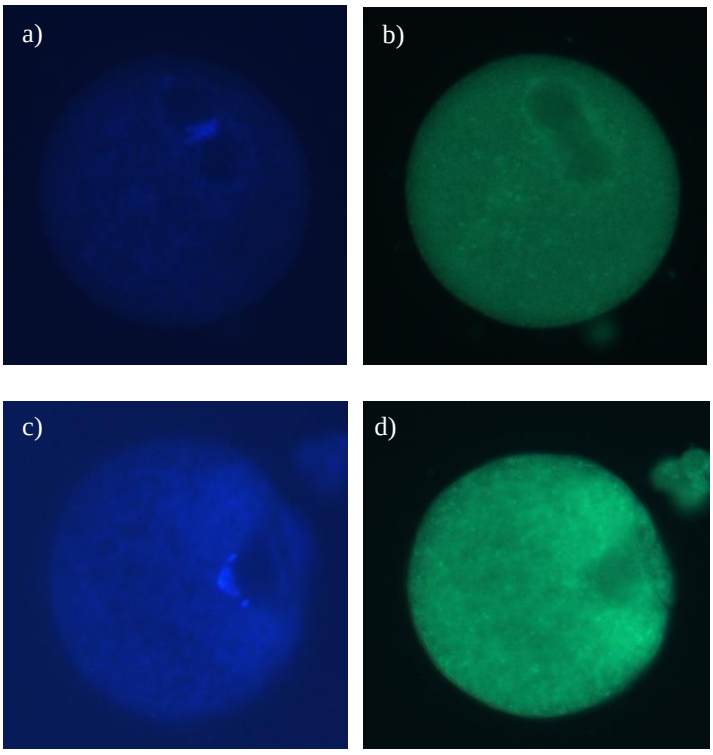


Figure 4 **Oocytes treated with TPEN for one hour during the 10 hour incubation period showed the chromosomes close to metaphase I (a) with a zinc surrounding the meiotic spindle and forming a bubble (b). One of the oocytes imaged displayed misaligned chromosomes (c) which appear to be pulled to one side of the oocyte without proper separation (d).**

The experiment was repeated with an incubation period of 8 hours. After 8 hours, the control oocytes appeared to have the chromosomes either lining up or beginning to separate but it was impossible to tell which one was occurring (Figure 5a). Again, a bubble outlined with zinc appeared to surround the meiotic spindle (Figure 5b). The oocytes treated with TPEN for 1 hour showed similar results and it was impossible to tell if the chromosomes were lining up during metaphase I or beginning to separate in anaphase I (Figures 5c, 5d).

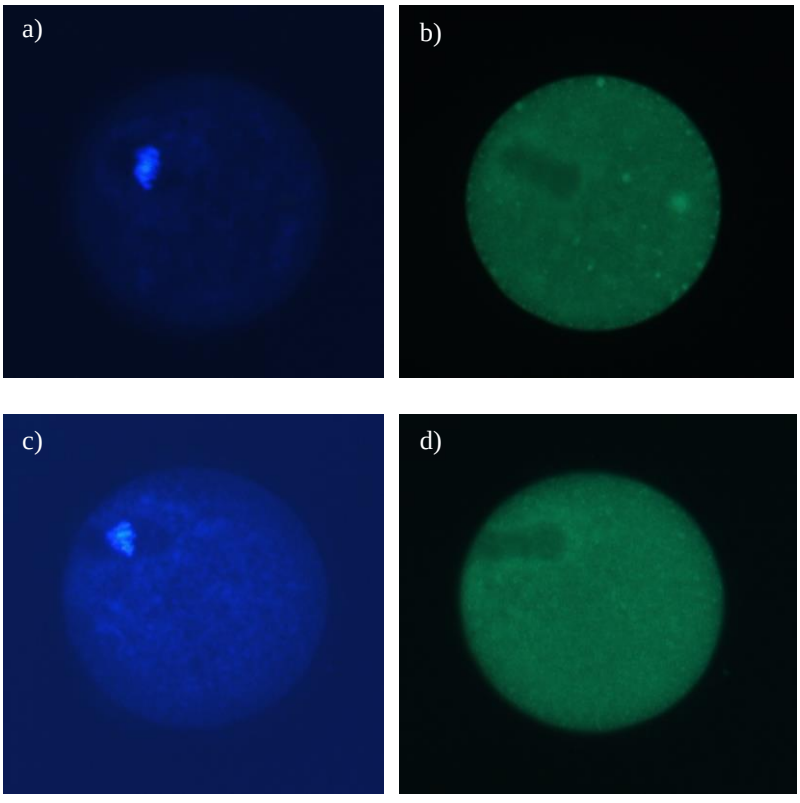


Figure 5 **After 8 hours oocytes untreated with TPEN showed chromosomes either lining up or beginning to separate (a). A bubble outlined with zinc can be seen surrounding the meiotic spindle (b). Oocytes treated with TPEN showed similar results after 8 hours (c,d).**

An uneven distribution of zinc occurs in aged oocytes

After imaging, a comparison of oocytes from 3 week old mice and oocytes from 8-9 month old mice was made. Oocytes from young mice showed an even distribution of intracellular zinc throughout the cytoplasm (Figure 6a). Oocytes from aged mice showed an uneven distribution of intracellular zinc with the zinc appearing to be contained in vesicles throughout the cytoplasm (Figure 6b).

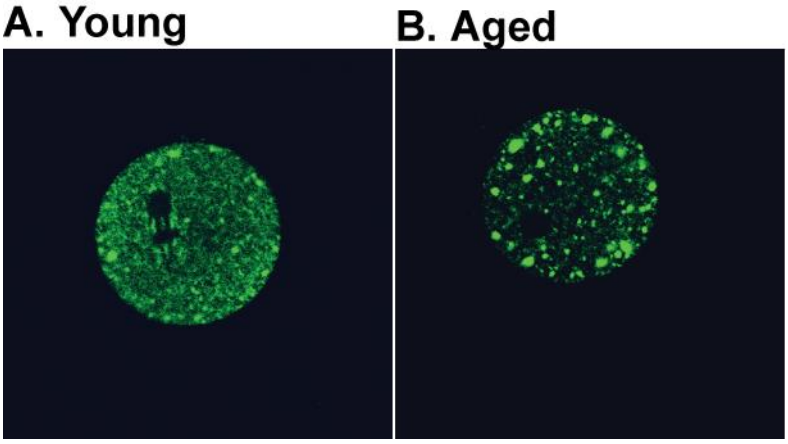


Figure 6 **Oocytes from 3-week old mice (a) showed an even distribution of intracellular zinc throughout the cytoplasm. Oocytes 8-9 month old mice (b) showed a patchy distribution of intracellular zinc, which appeared to be contained in vesicles throughout the cytoplasm.**

Discussion

Paracine signaling between cumulus cells and oocytes is important for proper development and maturation of oocytes. Cumulus cells promote developmental competence of oocytes by supplying the oocyte with nutrients (Eppig et al. 2005, Sugiera et al. 2007, Su et al. 2008), inducing transcriptional silencing (De La Fuente and Eppig 2001) and preventing resumption of meiosis before ovulation (Pincus and Enzmann 1935, Zhang et al. 2010). Studies have recently shown that transition metals may play very important roles in oocyte development and maturation. Zinc, in particular, is important for completion of meiosis (Kim et al. 2010, Bernhardt et al. 2011) and establishment of metaphase II arrest (Suzuki et al. 2010b, Kim et al. 2011). This could have great implications for reproductive quality of oocytes.

This study has shown that zinc plays a specific role during meiotic progression. Zinc appears to be important during metaphase or during the metaphase-anaphase transition during both meiosis I and meiosis II as it forms a bubble around the meiotic spindle. It is possible that the zinc plays a role in correctly aligning the chromosomes during metaphase or that the zinc has a role in attaching the spindle fibers to the centromeres of the sister chromosomes or chromatids to enable separation of the DNA during anaphase. This would explain why one of the oocytes incubated with TPEN during the ten hour period displayed an improper separation of the chromosomes. Additional research would need to be performed to answer this question.

This study also explored the effects of SAM rescue on the maturation rate of zinc deficient oocytes. While the control oocytes appeared to be aided by the addition of SAM, zinc deficient oocytes did not appear to show much effect with the addition of SAM. Therefore, it was

concluded that SAM could not rescue the oocytes collected from mice fed a zinc deficient diet. It is possible that the zinc deficient oocytes may have spindle defects, which could explain why the SAM had more effects on the control oocytes than the zinc deficient oocytes. Zinc deficiency could be a major concern for reproductive success, especially if zinc is required for proper oocyte development as indicated by the previous studies (Kim et al. 2010, Tian and Diaz 2012, Tian and Diaz 2013).

Aged mice also had significant observable differences in the levels of free intracellular zinc compared to young mice. Since an increase in zinc is required for completion of the first meiotic division (Kim et al. 2010), the low levels of intracellular zinc in aged oocytes could have effects on subsequent chromosome separation. Low levels of zinc in aged oocytes could also explain why these oocytes are more prone to aneuploidy than oocytes from young individuals (Merriman et al. 2012). This could have great implications for reproductive therapies for older individuals, as increasing zinc levels may regulate zinc-mediated meiotic pathways and improve fertility.

Assisted reproductive therapies utilize hormonal stimulation and in vitro manipulations to target the periovulatory transition.Problems during oocyte maturation can affect development and postnatal health (Doherty et al. 2000, Mann et al. 2004, Rivera et al. 2008). Regulation of zinc homeostasis in the oocyte is quickly becoming a parameter of oocyte quality (Kim et al. 2011, Lisle et al. 2013). This type of knowledge may be applicable to in vitro maturation (IVM) processes and could potentially increase the success of human IVM.

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