

**EMBRYONIC DEVELOPMENT OF IN VITRO MATURED
MOUSE OOCYTES FOLLOWING VITRIFICATION AND
IN VITRO FERTILIZATION**

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Abstract

With increasing incidences of early onset of cancer and the delay of reproductive age, the demand for fertility preservation in women have increased. Even though most results of oocyte cryopreservation have been obtained from ovarian stimulation protocols for in vitro fertilization (IVF), ovarian stimulation with gonadotropins may not be suitable for many women, especially those who are with hormone-dependent cancers, or those require immediate chemotherapy. However, it is agreed that the outcome of in vitro matured (IVM) oocytes are not as good as in vivo matured ones. In this thesis, we tested the interfering of oocytes during IVM, by the addition of antioxidant and the blocking of mitochondrial function, and observed their survival and the outcome of embryonic development after vitrification and IVF. For the first part, our study showed that the presence of the antioxidant, cysteamine during IVM significantly ($P<0.05$) reduced the fragmentation rate in cumulus-intact group from 61.9% to 40.8% following vitrification-thawing and IVF. However, the same trend wasn't shown in denuded groups. Nevertheless, there were no significant differences in terms of maturation and cleavage rate with the supplementation of cysteamine in either COC or denuded groups. We hypothesized that cysteamine facilitated effect during IVM might have occurred by affecting mtDNA accumulation during IVM. Fluorescent real-time quantitative analysis of mtDNA showed that the mtDNA copy number were similar in cysteamine treated and untreated group with a ratio close to 1, which implied that the effect of cysteamine during IVM may not be directly related to the mtDNA replication. In the second part, a mitochondria blocker, rotenone, was added to the IVM medium in order to test the

vitrification outcome and the subsequent embryonic development. The testing of rotenone concentrations from 100nM to 50µM, showed a lethal dose of around 50µM where all the oocytes were lysed during IVM. With the increase of rotenone concentration from 2µM to 50µM, the oocyte maturation rate dropped significantly in a dose dependent manner and eventually reaching 0 when the concentration was at 50µM. Within the concentration window of 250nM to 2µM, where no difference has been found in terms of the oocyte death and maturation rates compared to the control, there is a significant drop of cryo-survival rate at a concentration of 2µM, from 93.3% (in the control) to 55.6%. The percentage of embryos that developed to 4-8 cell stage were significant lower in the 2µM group (17.5%), compared to the group with a lower concentration of 250nM (50%). This study confirmed the strong positive dependency of mitochondrial functionality during oocyte maturation on its later development as well as the cryopreservation outcomes. Our work suggested that as two individual ART applications, the understanding and improvement of IVM could improve the outcome of vitrification since the impact of IVM on the oocytes could significantly determine the oocytes ability to survival and to develop following vitrification and IVF.

Résumé

Suite à l'augmentation de l'incidence des pathologies cancéreuses chez les jeunes et au recul de l'âge parental, les demandes de préservation de la fertilité sont de plus en plus fréquentes. Bien que la majorité des résultats sur la cryopréservation des ovocytes aient été obtenus suite à un protocole de stimulation ovarienne dans le cadre d'un traitement par fécondation *in vitro* (FIV), l'administration de gonadotrophines est inappropriée pour un certain nombre de patientes, en particulier celles atteintes de cancer hormono-dépendant, ou celle nécessitant une chimiothérapie sans délai possible. D'autre part, il est bien établi que le taux de fécondation des ovocytes maturés *in vitro* (IVM) n'est pas aussi élevé que lorsque les ovocytes sont maturés *in vivo*. Dans cette thèse, nous avons testé l'effet de la présence d'antioxydants et d'inhibiteurs de la fonction mitochondriale dans le milieu de maturation *in vitro* sur le taux de maturation ovocytaire (IVM) ainsi que sur le taux de survie et de développement embryonnaire après vitrification.

Dans un premier temps, nous avons démontré que la présence d'antioxydants, la cysteamine, pendant l'IVM réduit significativement le taux de fragmentation après vitrification des ovocytes maturés avec leur cellules du cumulus (complexes ovocyte-cumulus, COC), de 61.9% dans le groupe non traité à 40.8% dans le groupe traité ($p < 0.05$). Cette différence n'est pas observée dans le groupe des ovocytes maturés en l'absence de leur cellules du cumulus (ovocytes dénudés). La présence de cysteamine n'a pas d'effet significatif sur le taux de maturation et de clivage embryonnaire que les ovocytes soient dénudés ou non.

Nous avons ensuite vérifié si l'effet bénéfique de la cysteamine durant l'IVM était lié à

l'accumulation de DNA mitochondrial durant la maturation ovocytaire. Le nombre de copies d'ADN mitochondrial dans les ovocytes maturés en présence ou non de cysteamine a donc été évalué par PCR quantitative et le résultat ne montre pas de différence significative entre les deux groupes (ratio ~1). Ces données ne sont donc pas en faveur d'un effet de la cysteamine sur la réplication de l'ADN mitochondrial lors de la maturation ovocytaire *in vitro*.

Dans un deuxième temps, un inhibiteur de la fonction mitochondriale, la rotenone, a été ajoutée au milieu de maturation *in vitro* afin de tester son effet sur la vitrification et le développement embryonnaire ultérieur. Les tests d'échelonnement de concentrations, de 100nM à 50µM, ont permis de définir une dose létale de 50µM, pour laquelle la totalité des ovocytes sont lysés. Entre 2µM et 50µM, le taux de maturation diminue de manière dose dépendante, atteignant 0 à la dose maximale. Dans une échelle de concentration allant de 250nM à 2µM, aucune différence significative n'est observée en terme de taux de survie et de maturation entre le groupe traité et le groupe contrôle. Par contre le taux de survie après vitrification diminue en présence de 2µM de rotenone dans le milieu IVM, passant de 93.3% dans le groupe contrôle à 55.6% dans le groupe traité. Le taux d'embryons atteignant 4-8cellules après fécondation est également significativement plus bas dans le groupe traité à une concentration de 2µM comparé à un groupe traité à une dose plus faible de 250nM (respectivement 17.5% et 50%).

Cette étude confirme le rôle essentiel des mitochondries durant la maturation ovocytaire, le développement ultérieur et la survie après cryopréservation. Notre travail montre l'interaction entre deux techniques utilisées en Procréation Médicalement Assistée : la

maturation *in vitro* d'une part et la vitrification d'autre part. La compréhension et le développement des milieux de maturation ovocytaire *in vitro* ont en effet un impact majeur sur la survie des ovocytes après vitrification et sur leur développement embryonnaire ultérieur.

Abbreviations

ART: Assisted Reproductive Techniques

CAT: Catalase

COC: Cumulus Oocyte Complex

EG: Ethylene Glycol

FBS: Fetal Bovine Serum

FSH: Follicle Stimulating Hormone

GSH: Glutathione

GV: Germinal Vesicle

GVBD: Germinal Vesicle Break Down

HCG: Human Chronic Gonadotrophin

IVF: In vitro Fertilization

IVM: In vitro Maturation

LH: Luteinizing Hormone

MI: Meiosis I

MII: Meiosis II

mtDNA: Mitochondrial DNA

OHSS: Ovarian Hyperstimulation Syndrome

OS: Oxidative stress

PCOS: Polycystic Ovary Syndrome

PMSG: Pregnant Mare Serum Gonadotrophin

POLG: polymerase γ

PROH: 1,2-propanediol

ROS: Reactive oxygen species

SOD: Superoxide Dismutase

TFAM: Mitochondrial transcription factor A

XOD: Hypoxanthine-xanthine oxidase

Chapter 1. Introduction

With increasing incidences of early cancer onset and the delay of reproductive age, the demand for fertility preservation in women have increased. Even though most results of oocyte cryopreservation have been obtained from ovarian stimulation protocols for in vitro fertilization (IVF), ovarian stimulation with gonadotropins may not be suitable for many women, especially those with hormone-dependent cancers, or those who require immediate chemotherapy. Hence, oocytes have to be matured in vitro followed by vitrification. A study has shown that in vitro matured oocytes are more prone to cryo-damage and result in lower cleavage and blastocyst rate. (Succu et al., 2008) We believe that the conditions of oocytes maturation will have critical effects on its vitrification outcome. The works presented in this thesis were focused on the impact of oxidative stress and the disturbance of mitochondria function during IVM on the cryopreservation and the fertilization outcome of those IVM oocytes. To do so, the first part was performed by supplementing an antioxidant into the maturation medium during IVM, followed by vitrification. Mitochondrial DNA (mtDNA) copy number was tested at the end of maturation to see if the change of mtDNA replication was an underlying mechanism resulted by the change of redox state upon cysteamine supplementation. In the second part, a mitochondrial blocker was added during IVM in order to disturb mitochondrial function. Embryonic development competency was assessed based on the fragmentation, fertilization and blastocyst formation rates after fertilization in those IVM oocytes. Overall, this thesis will attempt to answer three specific questions regarding the

change of condition during IVM on the cryopreservation outcome of vitrified oocytes.

1. Will the supplementation of antioxidant during IVM facilitate the early embryonic development in vitrified oocytes?
2. Will the change of redox state during IVM affect the mtDNA copy number?
3. Is mitochondrial function during IVM related to the cryopreservation outcome of MII oocytes?

1.1 The need to merge IVM technique with vitrification:

Vitrification is an alternative method to traditional slow freezing method that directly transfers oocytes to a glass state under the presence of cryoprotectants to avoid chilling injury and ice crystal formation. As the increasing incidences of early onset of cancer, cancer survivors who are still at their reproductive age may have impairment of their fertility resulted from the cancer treatments. Cryopreservation of their oocytes before cancer treatment could help to preserve their fertility so they would still be able to have their biological children if surviving cancer treatments. Even though most results of oocyte cryopreservation have been obtained from ovarian stimulation protocols for IVF, ovarian stimulation with gonadotropins may not be suitable for many women who need to preserve their fertility, especially those with hormone-dependent cancers, or those who require immediate chemotherapy. Also, for poor hormonal stimulation responders, the number of mature oocytes obtained is usually limited; hence any immature oocytes are needed to extend the pool of fertilizable oocytes (Chang et al., 2008). In both cases, oocytes will be matured in vitro followed by vitrification.

1.2 Oxidative stress during IVM and antioxidant supplementation

Reactive oxygen species (ROS) are formed as intermediary products of cellular metabolism. They could cause structural and functional alternation of the cells due to its oxidative property. During IVM, oocytes are extremely vulnerable to oxidative stress not only from the cellular metabolism, but also external influences, such as light and rapid change of oxygen concentration. Intracellular redox status is one factor that may influence the ability of cells to survive and its developmental competence after vitrification. Cysteamine has been used as supplementation during both oocyte maturation and embryo development. It increases oocytes/embryos antioxidative capacity via raising intracellular level of Glutathione (GSH)(Bannai, 1984, Issels et al., 1988). It has been demonstrated that cysteamine supplementation in IVM medium of mouse oocytes increased the intracellular GSH content, and subsequently improved in vitro blastocyst development (de Matos et al., 2003). In our study, cysteamine was supplemented into the maturation medium as an antioxidant to change the redox state of oocytes during IVM.

1.3 Mitochondria as a target for oocyte quality

While the role of mitochondria in ATP production has long been known, their contribution to oocyte and embryo competence has only recently been studied. It is believed that mitochondria function, activity and distribution are critical in establishing and maintenance of developmental viability of the oocytes, especially during oocyte maturation, which is a highly energy dependent process. In other word, the functional level of mitochondria could be an indicator for oocyte cytoplasmic maturation, hence

directly related to the competency of later embryonic development. It has been shown that the transfer of ooplasm from younger to older eggs benefits in rejuvenating fertility in older women, and to treat the age-related cytoplasmic dysfunction responsible for oocyte aneuploidy (Zhang et al., 1999, Takeuchi et al., 2001).

1.4 Hypotheses:

Hypothesis 1: The supplementation of antioxidant during IVM could benefit the oocytes ability to resist cryo-damage after vitrification.

Hypothesis 2: The underlying mechanism of the long term effect of cysteamine during IVM involves the changing of mtDNA accumulation during IVM.

Hypothesis 3: Mitochondrial function is crucial for the recovery and subsequent fertilization and embryonic development of oocytes after vitrification and IVF.

1.5 Thesis outline:

The first chapter will introduce the research topics. The second chapter is a review of the related literature, the rationale behind our works and what has been accomplished so far. The first concept is to test the effect of antioxidant supplementation during IVM on the cryopreservation outcome and the possible underlying mechanism. The second concept is to test if the disturbance of mitochondria during IVM affects the outcome of vitrification. The core of this thesis, chapter 3 and 4 contains two manuscripts, one of which has been presented in ASRM (American Society of Reproductive Medicine) meeting. Finally, in chapter 5, we will summarize the results obtained in the two studies, and what is more can

be done in the future.

Chapter 2: Background and Literature Review

2.1 In vitro maturation and vitrification

2.1.1 In vitro maturation of oocytes

In each menstrual cycle of women, cohorts of oocytes commence growth, and only one of them gains dominance, growing to germinal vesicle stage under the influence of follicle stimulating hormone (FSH). With the luteinizing hormone (LH) surge, oocyte undergoes germinal vesicle breakdown (GVBD) and undergo meiotic division coupled by the extrusion of first polar body. Oocyte maturation is defined as the completion of the first meiotic division from germinal vesicle (GV) to metaphase II (MII) stage. Under ovarian stimulation, couple of matured oocytes (MII) could be retrieved from the patients, which could undergo fertilization to form embryos. However, there are circumstances when limited number of matured oocytes can be obtained, and in vitro maturation technology is essential to extend the pool of total fertilizable oocytes. For example, patients with poor response to hormonal treatment, patients at high risk of ovarian hyperstimulation syndrome (OHSS); or those who can't undergo hormone treatment. The first pregnancy from eggs that were obtained from an ovary at the time of a cesarean section and matured in vitro was reported at 1991 by Cha et al.,(Cha et al., 1991) In vitro maturation IVM has been proven to be an effective method for patients with polycystic ovary syndrome (PCOS)(Chian et al., 1999, Holzer et al., 2007), and may be used as a new strategy to combine natural-cycle IVF with IVM of immature oocytes(Chian et al., 2004). However,

IVM remains to be an experimental procedure, due to its relatively low successful outcome.

2.1.2 **Oocyte vitrification**

Oocyte vitrification, also referred as rapid-cooling method is a relatively new method in gamete cryopreservation. During vitrification, water is cooled at an extremely rapid speed, and with the addition of cryoprotectants which prevents ice crystal formation, cells would be ideally left with no structural damage by ice crystal formation during cooling. Vitrification is superior to the conventional slow-freezing method by its relatively high survival rates, and better embryonic development (Hong et al., 1999, okimura T, 2005, Lucena et al., 2006). The first successful human live birth with vitrified in vivo matured oocytes was reported in 1999, conceived with a vitrified matured oocyte(Kuleshova et al., 1999).

2.1.3 **Vitrification of IVM oocytes**

Theoretically, immature oocytes should be more resistant to damages caused during freezing and thawing since the chromatins are diffused and surrounded by a nuclear membrane(Cooper et al., 1998, Cao and Chian, 2009). However, poor maturation, fertilization and embryonic development were found with vitrified immature oocytes (Toth et al., 1994a, Toth et al., 1994b). While IVM of immature oocytes after frozen-thawing remains to be a challenge, cryopreservation of mature oocytes is agreed to be more effective than freezing immature oocytes for most species(Cao and Chian, 2009). Hence, immature oocyte retrieval followed by IVM and vitrification will be a promising

strategy to preserve fertility. Although several studies reveal no differences in oocyte survival rates and meiotic spindle, as well as chromosome alignment between in vivo and in vitro matured oocytes followed vitrification-thawing(Huang et al., 2008) (Chang et al., 2008). Clinical studies indicated that in vitro matured oocytes are more sensitive to cryopreservation and relatively lower survival rates were obtained after vitrification-thawing compared to in vivo matured oocytes(Chian et al., 2009, Chian et al., 2008).

2.2 Oxidative stress in oocytes

2.2.1 Production of ROS and oxidative stress

ROS are formed as intermediary products of normal cellular metabolism and also from external sources. The cell is under oxidative stress (OS) whenever the balance between pro-oxidant and antioxidant is disturbed, which may resulted from an increase of superoxides and free radicals, or a decrease of antioxidant, so that the cell's defense mechanism can no longer cope with the oxidative species formed. With the oxidative propriety of ROS, they could oxidize proteins, lipids and DNA, causing structural and functional alternation of the cell. When the oxidative stress is extended to a certain level, it may induce the release of cytochrome c and other apoptogenic factors from mitochondria which eventually activate programmed cell death(Orrenius et al., 2007).

2.2.2 The intrinsic oxidative defense systems in the oocytes

Oxidative defense system in the oocytes and embryos includes both enzymatic system

and non-enzymatic system. Enzymatic system is composed of superoxide dismutase (SOD), catalase(CAT), glutathione(GSH), and peroxiredoxins, which detoxifies ROS directly. Non-enzymatic system contains mainly vitamin A, C and E (Guerin et al., 2001, Dumollard et al., 2009). The transcripts encoding those enzymatic antioxidants such as GPx, Cu/Zn-SOD Mn-SOD and gamma-glutamylcysteine synthetase which is essential for GSH synthesis, have been shown to present in MII oocyte cross different mammalian species with minor variations(Combelles et al., 2009). On the other hand, the amount of those enzymatic antioxidant transcripts might be a reflection of the oxidative status of the oocytes under different conditions. For example, the transcript abundance of IVM oocytes was significantly higher than in vivo matured bovine oocytes(Lonergan et al., 2003).

2.2.3 **Influence of oxidative stress on IVM**

During IVM, oocytes are extremely vulnerable to oxidative stress due to the generation of ROS not only from cellular metabolism, but also external influence, such as light, rapid change of oxygen concentration, and the significant amount of oocyte handling. Studies in the bovine model suggested that transcript abundance for three antioxidative genes, Mn-SOD, Cu/Zn-SOD and sarcosine oxidase, were increased significantly in IVM oocyte, and this may signify an induction of oxidative defense in order to cope with the increase oxidative stress in in vitro culture(Lonergan et al., 2003).

2.2.4 **Oxidative stress related cryo-damage**

There has been considerable amount of studies done on the involvement of oxidative

stress in cryo-damage in sperm, whereas little is known in mammalian oocytes and embryos(Tatone et al., 2010). It has been shown that free radicals were formed during cryopreservation of tissues(Whiteley et al., 1992), which may be resulted from the change of temperature and osmotic damage in the cells(Thomson et al., 2009). Microarray analysis of freezing and thawing induced genes revealed that eukaryotic cells express different genes at various time points after warming in the categories of energy metabolism, oxidative stress scavengers, cell rescue system including heat shock proteins, and gene products localized in organelles(Odani et al., 2003, Tatone et al., 2010). It indicates the activation of cellular oxidative defense systems in response to damage produced upon vitrification. In addition to the increase of pro-oxidants, the cell's antioxidative defense system may be disturbed during the process of cryopreservation, such as the damage of antioxidant enzyme and antioxidant gene transcription which will alter the antioxidant enzyme potential of oocytes. Mouse embryos cryopreserved at the two-cell stage have elevated levels of intracellular H_2O_2 , which is known to induce developmental blockage of mouse embryos(Ahn et al., 2002). Analysis of GSH level in vitrified porcine oocytes revealed that the intracellular level of GSH in vitrified oocytes was lower than in the control oocytes, which suggested cooling and vitrification induces GSH loss in oocytes which might result in the embryos to be more vulnerable to oxidative damage(Somfai et al., 2007).

2.3 GSH antioxidant system and its effect when supplemented in the medium

2.3.1 Role of GSH

Glutathione (GSH) is a triple peptide, composed by three components cysteine, glutamate and glycine. Since it cannot enter the cell directly, it has to be made inside the cell from its three constituent amino acids. The rate limiting step of GSH synthesis is the incorporation of cysteine, hence the availability of cysteine is a determining factor for GSH synthesis (Meister, 1983). GSH has been known to participate in various mechanisms, such as microtubule assembly (Deneke and Fanburg, 1989), sperm decondensation and male pronuclear formation in different species (Funahashi et al., 1999, Yoshida et al., 1993, Sutovsky and Schatten, 1997), and most importantly, protect cells from oxidative damage by acting as reactive oxygen species scavengers (Meister, 1983). In the GSH, the sulfhydryl (thiol) group of cysteine serves as a proton donor and is responsible for the biological activity of glutathione. GSH has been considered to serve as a natural reservoir of reducing power and used extensively as a biomarker of OS by the ratio between its oxidized form and reduced form.

2.3.2 **GSH content during oocyte maturation and early embryonic development**

During maturation and development of the oocytes in the ovary, GSH content increases as the oocytes approach the time of ovulation. GSH concentration in ovulated MII oocytes is approximately the twice as high as in GV stage oocytes in hamster oocytes (Zuelke et al., 2003). After ovulation, early embryonic development is accompanied by a massive oxidation of GSH, with a 55% decrease at the time of zygotic genome activation, and decreases approximately 10-fold during pre-implantation development from the unfertilized oocytes to the blastocyst (Gardiner and Reed, 1994, Lubarda, 2005).

Concomitant to the rapid decrease of GSH, NADPH level also drop around 45%, which results a shift of intracellular redox state to oxidation(Dumollard et al., 2007). Hence, the accumulation of GSH in the oocytes during development is essential for protecting it in the later stages of fertilization(Telford et al., 1990). This is also supported by the findings that the impairment of the accumulation of GSH during oocyte maturation severely undermines the oocytes developmental potential (Luberda, 2005, Dumollard et al., 2009).

2.3.3 **Cysteamine supplementation facilitated increase of GSH content**

Supplementing cysteine directly in the medium is not efficient to stimulate GSH synthesis since cysteine can be easily oxidized to cystine (cysteine dimer) in the culture medium(Bannai, 1984). Oocytes have limited ability to uptake cystine. Cysteamine is a low molecular weight thiol compound. It has been used to promote intracellular GSH synthesis through promoting the cystine uptake into the cell. It reacts with cystine to form a mixed disulfide with its cysteine form, and being taken into the oocyte by a transporter system(Meier and Issels, 1995). Upon entering the oocytes, the mixture is reduced back to cysteine and thiol compounds. The thiol compound in its reduced form will be recycled to the medium and repeatedly taken up by the oocytes in the form of mixed disulfide with cysteine(Meier and Issels, 1995).

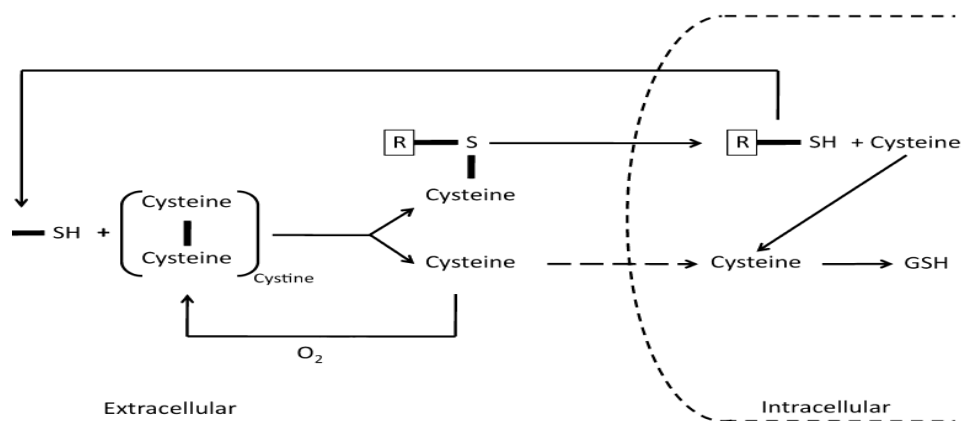


Fig 2.1 Cysteamine facilitated cysteine uptake in oocyte. Taken from *Cysteamine supplementation of in vitro maturation medium*, Delduze and Goudet, 2010. Cysteamine reacts with cystine outside of the cell to break its dimer form by forming a disulfide bond with its cysteine units and facilitated its transport into the oocytes. Upon entering the oocytes, the mixture is reduced back to cysteine and the thiol compound. The thiol compound in its reduced form will be recycled to the medium and engaged into the whole process again.

2.3.4 **History of the supplementation of cysteamine in maturation medium**

The beneficial effect of supplementation of cysteamine to the IVM medium has been studied in bovine (de Matos et al., 1995, de Matos et al., 1996, de Matos et al., 2002a, de Matos et al., 1997, Balasubramanian and Rho, 2007), buffalo (Gasparrini et al., 2000, Anand et al., 2008), goats (Rodriguez-Gonzalez et al., 2003b, Urdaneta et al., 2003), pigs (Kobayashi et al., 2006, Song and Lee, 2007, Bing et al., 2001), cats (Bogliolo et al., 2001), dogs (Hosseini et al., 2007) and mice (de Matos et al., 2003, Chen et al., 2005). The concentration of cysteamine supplementation used in those studies generally ranged from 25 μ M to 500 μ M with an optimal effective dose between 100 μ M to 200 μ M depending on the species and culture environments. It suggests that the optimal concentrations of cysteamine supplementation differs among species, and varies in culture environments.

2.3.5 **The effect of cysteamine supplementation: oocyte maturation, pronuclear formation, and blastocyst development**

The effects of cysteamine supplementation during IVM have been reported differently among studies. As reviewed by Deleuze (Deleuze and Goudet, 2010), studies on dogs (Hosseini et al., 2007), mice (Chen et al., 2005), goats (Urdaneta et al., 2003) and pigs (Bing et al., 2001) showed improved nuclear maturation rates, while other studies in goats (Zhou et al., 2008), pigs (Yamauchi and Nagai, 1999, Kobayashi et al., 2006, Song

and Lee, 2007), horses(Luciano et al., 2006, Deleuze et al., 2010), bovine (Oyamada and Fukui, 2004, Balasubramanian and Rho, 2007) didn't show any significant increase in oocyte nuclear maturation rates. This discrepancy may be due to the variation of experimental environments between studies, such as medium composition, oocytes handling. Hence, it is difficult to conclude the approximate effect of cysteamine supplementation on the oocyte nuclear maturation rate.

It has been pretty consistent between studies that the addition of cysteamine to IVM medium has improved male pronuclear formation following IVF, in goats(Rodriguez-Gonzalez et al., 2003a, Urdaneta et al., 2003), buffalos(Anand et al., 2008) and pigs(Gruppen et al., 1995, Yamauchi and Nagai, 1999, Bing et al., 2001). This could be achieved by enhancing the decondensation of male pronucleus, or through reducing oxidative stress and improving cytoplasmic maturation, which facilitate male pronuclear formation(Tatemoto et al., 2004, Deleuze and Goudet, 2010).

Most studies have shown an increased blastocyst development with cysteamine supplementation to the IVM medium at various concentrations, such as in bovine (Takahashi et al., 1993, de Matos et al., 1995, de Matos et al., 2002b, Oyamada and Fukui, 2004, Balasubramanian and Rho, 2007), buffalos(Gasparrini et al., 2003, Anand et al., 2008, Singhal et al., 2009), sheep(de Matos et al., 2002a), goats(Zhou et al., 2008), pigs(Gruppen et al., 1995, Kobayashi et al., 2006) and mice(de Matos et al., 2003), while a few reported no significant difference, in pigs(Kobayashi et al., 2007, Song and Lee,

2007), horse (Deleuze et al., 2010), and mice (Chen et al., 2005), reviewed by Deleuze and Goudet (Deleuze and Goudet, 2010).

2.3.6 **The effect of cysteamine supplementation on the resistance of cryo-damage**

Even though cysteamine has been broadly used as supplementation for in vitro embryo production, little has been known about the contributing effect of antioxidant on the cryopreservation outcome of the oocytes/embryos. A recent study on cysteamine supplementation on embryonic cryopreservation outcome showed that cysteamine supplementation of culture medium during embryonic development before vitrification and/or after vitrification-warming improved overall survival of frozen-thawed embryos (Hosseini et al., 2009).

2.3.7 **Role of cumulus cells on the cysteamine supplementation**

It has been proposed by many studies that cumulus cells play important roles in protecting cells from oxidative stress during oocyte maturation. In Tatemoto et al. (2000) study, COC and denuded oocytes were treated with hypoxanthine-xanthine oxidase (XOD) to induce oxidative stress. As a result, denuded oocytes showed greater DNA nuclear damage and apoptotic degeneration compared to COC. The GSH level in denuded oocytes were significantly reduced irrespective to the presence or absence of XOD system (Tatemoto et al., 2000). Looking into the role of cumulus in facilitating GSH synthesis, studies have been done to test how glutathione level changes responding to the addition of cystine, cysteine, and cysteamine in the presence or absence of cumulus cells. De matos et al. (1997) reported that the increase of GSH level by cystine was only observed in the

presence of cumulus cells. Addition of cystine with cysteamine to IVM medium could increase GSH level in both COC and denuded oocytes (de Matos et al., 1997). Zhou et al. (2008) also showed similar result using goat model that oocyte alone can't uptake cystine effectively unless supplemented together with cysteamine, while addition of cystine alone could be enough to improve the GSH synthesis in COCs (Zhou et al., 2008). Upon the addition of cysteamine, a significant increase of GPx mRNA was observed comparing to the absence of cysteamine (Luciano et al., 2006), which is concomitant to the facilitating role of cumulus in GSH synthesis in the oocytes. In both Luciano et al. (2005) and Zhou et al. (2008) study in goat and bovine showed that co-culture of denuded oocytes with cumulus cells (gap junction destroyed) during IVM could restore the developmental capability and GSH content in the oocytes to a certain extent, which suggests the presence of putative diffusible factor(s) produced by cumulus cells that facilitates GSH synthesis in the oocytes (Zhou et al., 2008, Luciano et al., 2005).

2.4 Mitochondria is a key factor in cytoplasmic maturation

2.4.1 Critical role of mitochondria during IVM

Mitochondria are the energy producing organelles that are essential for all cellular activities. The availability of functional mitochondria has been recognized as a critical determinant of human embryo development competence since the defects at the structure and mtDNA levels have been identified in compromised oocytes/embryos (Reynier et al., 2001).

In physiological condition, oocyte maturation is coupled by the distribution changes of active mitochondria. Mitochondria distribution shows a homogeneous pattern at GV stage, and adopts a heterogeneous distribution with mitochondria clustered around nucleus at M I and M II stage (Nishi et al., 2003). The same mitochondrial pattern during oocyte maturation have also been reported in cow (Stojkovic et al., 2001), pig(Sun et al., 2001) MII oocytes with abnormalities in mitochondria distribution, such as occurrence of unusual large aggregates has been shown to be negatively correlated with developmental competency(Wilding et al., 2001), Since the formation of meiotic spindle is highly ATP dependent, the hinder of mitochondria function could result in inappropriate formation of the cytoplasmic microtubule network.

2.4.2 **mtDNA in the oocytes**

Mitochondria genome is maternally inherited. All mitochondria in a mature oocyte arise from the clonal expansion of an extremely small number of mitochondria in the primordial germ cell (Van Blerkom, 2004). It replicates throughout oocytes growth but does not in the early cleavage stages of embryogenesis until blastocyst stage in mice (Piko and Taylor, 1987, Ebert et al., 1988), which means that the mtDNA has to be amplified to a sufficient level prior to fertilization.

Subnormal mitochondrial numbers has also been related to premature arrest of preovulatory maturation, abnormal organization(Reynier et al., 2001) and function of meiotic and mitotic spindles leading to chromosomal aneuploidy at MII stage

oocytes(Zeng et al., 2007). Significant lower mtDNA copy number has been found in the oocytes of the patients who have had repeated fertilization failure, (Reynier et al., 2001) and individual oocytes that couldn't be fertilized (Santos et al., 2006). It has been proposed that a critical threshold of 100, 000 copies in MII stage oocytes for them to be competent in embryonic development (Piko and Taylor, 1987).

2.4.3 **mtDNA replication during in vitro maturation**

During oocyte maturation, mtDNA replication has been reported in porcine (Spikings et al., 2007) and mouse oocytes(Piko and Taylor, 1987). Spikings et al,(2007) showed a significant increase in mtDNA copy number during porcine oocyte maturation, and the expression of mtDNA replication factors, polymerase gamma (POLG) and mitochondrial transcription factor A (TFAM). On the other hand, blocking the mtDNA replication diminished this mtDNA increase during IVM, which confirmed this active replication process during IVM (Spikings et al., 2007). Accumulation of mtDNA deletions demonstrated during oocyte maturation in the rhesus macaques oocytes (Gibson et al., 2005) also suggested the mtDNA replication took place during IVM.

2.4.4 **Big variation in mtDNA copy number**

Reviewing previous publication on mtDNA quantification, there has been a big variation on the reports of mtDNA copy numbers in a single oocyte. (Table 2.1) The early quantification with Dot blot analysis method revealed copy number of 119,000 in mouse oocytes(Piko and Taylor, 1987), and more recent PCR results reported a copy number of

157,000 in mouse (Cao et al., 2007). With Real time PCR method, Steuerwald et al., group reported a copy number of 314,000 (Steuerwald et al., 2000), Reynier et al. group showed a copy number of 209,000 (Reynier et al., 2001); and Chen et al group reported a copy number of 138,000 in single human oocyte. As indicated by several studies, there was a considerable variation between samples extracted from same individual as well. For example, Steuerwald et al., reported an average mtDNA copy number of 314,000, with a concentration range of among their samples from a low of 90,000 and high of 600,000 (Steuerwald et al., 2000). Hence, the difference in the mean mtDNA content of oocytes could be explained by the large inter-individual variations, as well as the oocytes belonging to the same cohort.

Table 2.1: mt DNA copy number in Matured oocytes

Method	Species	mtDNA copy/ oocyte	Author
Electron microscopy	mouse	92,500	Piko and Matsumoto, 1976
Dot blot analysis	mouse	119,000	Piko and Taylor., 1987
Real-time PCR	mouse	157,000	Cao et al.,2007
Hybridization	Bovine	260,000	Michaels et al.,1982
Real-time PCR	Human	138,000	Chen et al.,1995
Electron micrographs	Human	400,000	De Boer et al.,1999
Real-time PCR	Human	795,534	Barritt et al., 2002
Real-time PCR	Human	209,000	Reynier et al.,2001
Real-time PCR	Human	314,000	Steuerwald et al.,2000

2.4.5 **Impact of oxidative stress on mtDNA replication**

Only one study has shown that the variation of mtDNA copy number from fertilization to birth is associated with oxidative stress (Aiken, 2008).

2.5 **Mitochondria related meiotic spindle defects**

2.5.1 **The function of spindle is energy dependent**

Both MI and MII are highly energy-intensive processes. (Fig.2.2) ATP is essential for the function of motor proteins, Dynein and Kinesin to capture and transport chromosome along the spindle. Both the synapsed chromosome pairs in MI and sister chromatid in MII have to be pulled to the opposite ends of the dividing oocytes along the mitotic spindle which is guided by the extending and shortening of the meiotic spindles (Eichenlaub-Ritter et al., 1996). During oocyte maturation, mitochondria accumulate around the nucleus from its homogenous layout before the start of GVBD (Van Blerkom, 1991), which indicates the important role of mitochondria in energy production during oocyte maturation.

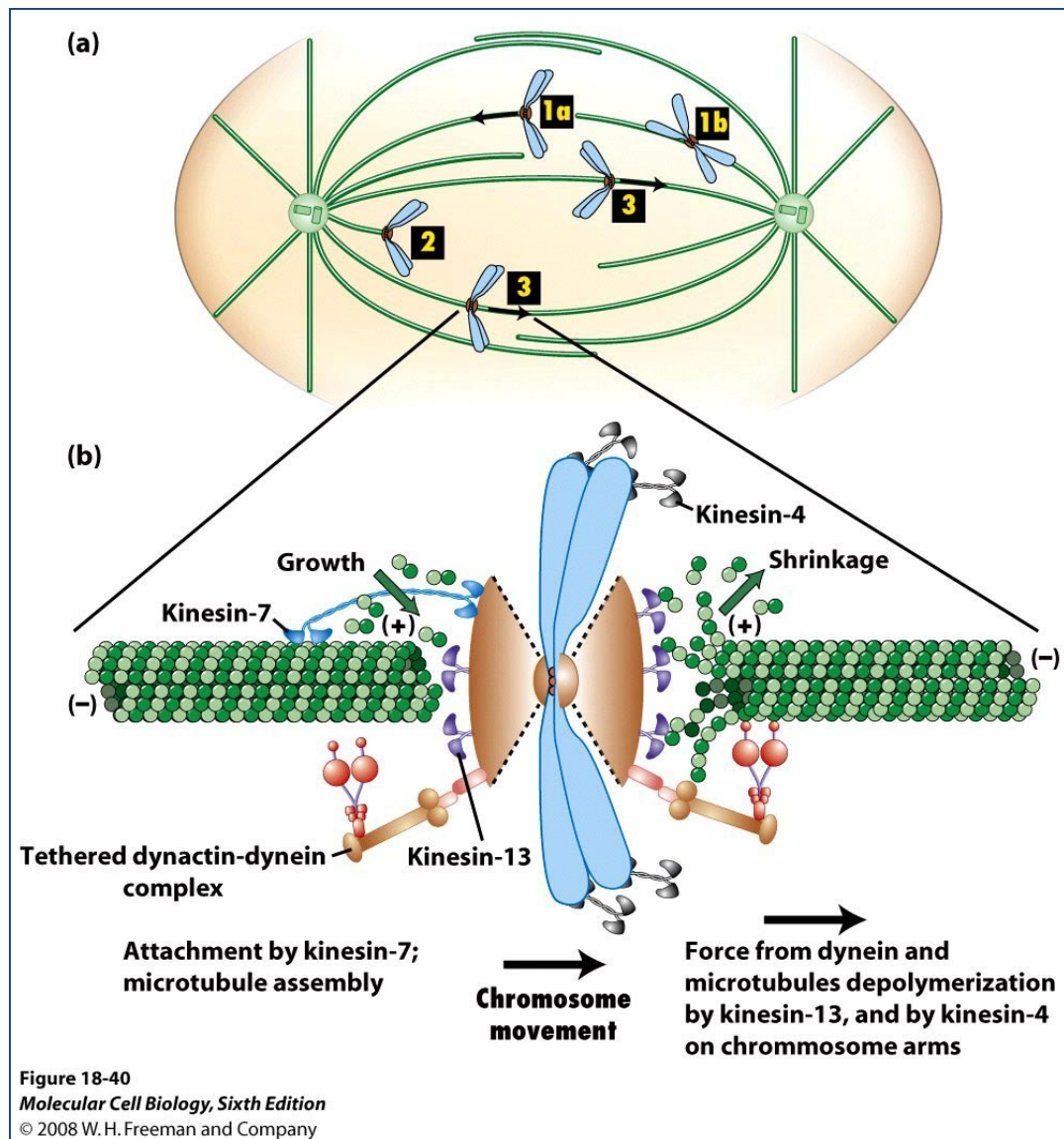


Fig2.2: mitotic spindle function during meiosis. Taken from *Molecular cell biology, sixth edition, 2008, W.H.Freeman and company*

Dynein and kinesin are motor proteins using ATP as fuel to capture and transport the chromosome along the microtubule toward the centrosomes.

2.5.2 **Aneuploidy in the events of MI and MII**

Both MI and MII errors increase with maternal age(Yoon et al., 1996). A “two-hit” process of non-disjunction has been suggested by E.A. Schon. The first hit is the distribution of chromosome for the pericentromeric recombination events per chromosome bivalent, which creates pair susceptible to non-disjunction. During the “second hit”- second meiotic division, oocytes are likely to undergo non-disjunction upon the presence of any other inducer factors, such as deteriorated mitochondria condition in older women, or damage resulted by cryopreservation(Schon et al., 2000).

2.5.3 **Energy deficiency related aneuploidy**

Metabolic rate and ATP content varies significantly between oocytes and embryos (Gott et al., 1990, Slotte et al., 1990). Study in human oocytes showed that oocytes from cohorts that have higher ATP content have higher potential for continued embryogenesis and implantation (Van Blerkom et al., 1995). While meiotic maturation, fertilization and early cleavage could still progress under reduced level of ATP, the morphologically normal embryos may not succeed in implantation due to the low ATP level firstly established in the oocytes (Van Blerkom et al., 1995).

In the oocytes, mitochondria has only 1 copy of the genome (Piko and Taylor, 1987) comparing to in the somatic cells which has an average of five mtDNAs per cells (Satoh and Kuroiwa, 1991). This property renders it very prone to oxidative damage and mtDNA mutations.

Aneuploidy tends to increase significantly with maternal age. The risk of trisomy pregnancy is around 2-3% in women among the age of early 20s, however, the risk can increase to 30-35% in women at their 40s (Hassold and Chiu, 1985). Oocytes in older women showed an accumulation of mitochondria point mutation, deletions, which results in respiratory chain defects and a compromised ATP production (Shigenaga et al., 1994, Barritt et al., 2000). Transfer of ooplasm from younger to older eggs benefits in rejuvenating fertility in older women, and to treat the age-related cytoplasmic dysfunction responsible for oocyte aneuploidy (Zhang et al., 1999, Takeuchi et al., 2001).

The idea of mitochondrial energy deficit resulted aneuploidy has been proposed long ago (Beermann and Hansmann, 1986) (Schon et al., 2000). Study done by Takeuchi et al, has shown that block of mitochondrial function inhibits GVBD and meiotic spindle formation, and transfer of germinal vesicle of treated GV oocytes to an enucleated healthy oocytes could restore its developmental competency to a normal level (Takeuchi et al., 2005).

2.6. The impact of vitrification on spindle integrity

2.6.1 Increased spindle abnormality in vitrified oocytes

Vitrified oocytes showed increased abnormalities in spindle and chromosome configuration, resulted by depolymerization of meiotic spindles and disappearance of microtubules organizing centers (Aman and Parks, 1994, Saunders and Parks, 1999, Webb et al., 1986). Those usually result lower cleavage and early embryonic development rates, in mice (Huang et al., 2008), and porcine (Wu et al., 2006). Fertilization of oocytes with

disrupted spindle could lead to aneuploidy, disgeny and arrested cleavage (Chen et al., 2003). During embryo freezing, large proportion of embryo that couldn't cleave after freezing and thawing cycle exhibit chromosome abnormalities (Laverge et al., 1998), and significantly decreased implantation rate (Gabrielsen A, 2004).

Vitrification requires the use of highly concentrated cryoprotectants, and the cryoprotectants may alter the structure of meiotic spindle. The effect of cryoprotectants on the meiotic spindles is reported to be dose dependent (Joly et al., 1992, Van der Elst et al., 1992). Early study found that the spindles of mouse oocytes showed disorganization when exposed to a vitrification solution of 5.5M ethylene glycol (EG) and 1.0 M sucrose immediately post-thawing, and partially repolymerized after 1 hour of incubation (Chen et al., 2000). In our study, vitrification medium contains 2.6 M EG, 2.0M 1,2-propanediol (PROH) and 1.0 M sucrose.

2.6.2 **Spindle behavior under low temperature**

Studies have shown that meiotic spindle in MII oocytes are very sensitive to low temperature. Microtubules disassemble partially or completely when oocytes were exposed to lower than room temperature (Wang et al., 2001, Almeida and Bolton, 1995, Aman and Parks, 1994). For human oocytes, around 50% spindle disassemble after 10 min at room temperature, and 100% after 30min at room temperature accompanied with reducing in the size of the spindle, and chromosome abnormalities (Pickering et al., 1990). After warming, the microtubules starts repolymerize during the 2-3 hours of incubation (Aigner et al., 1992, Chen et al., 2001), and could be restored in many, but not all of the

cases (Pickering and Johnson, 1987). A sufficient restoration of cytoskeleton after incubation is essential for chromatid segregation during second meiotic division and the polarity of the mature oocyte (Joly et al., 1992). Tan et al.(2009), has reported an optimal recovery time of 2 hours after thawing, which results significant higher fertilization rate than 1 hour and 3 hours groups (Tan et al., 2009).

Chapter 3 Experiment 1

Effect of cysteamine supplementation during oocyte IVM on subsequent embryo cleavage and development following vitrification and IVF

Summary:

Cysteamine has been widely used as a supplementation in the maturation medium due to its proven beneficial effect on the oocyte subsequent embryonic development after fertilization. However, the effect of cysteamine supplementation during oocyte IVM on the later embryonic development competency after vitrification has never been investigated. The purpose of this study is to determine the effect of cysteamine during oocyte IVM on their early embryonic developmental competency after vitrification, and the possible underlying mechanism. Our study showed that the presence of cysteamine during oocyte IVM significantly ($p<0.05$) reduced the fragmentation rate of embryos in cumulus-intact group from 61.9% to 40.8% following vitrification-thawing and IVF, but not in denuded groups. There was no significant difference in terms of oocyte maturation, embryo cleavage rate with the supplementation of cysteamine. Fluorescent real-time quantitative analysis of mtDNA showed that mtDNA copy number were similar in cysteamine treated and untreated group with a ratio close to 1, which implied that during oocyte IVM cysteamine supplementation had no effect on the regulation of mtDNA replication.

3.1 **Introduction:**

Fertility cryopreservation has become an indispensable part of ART. As the increased incidence of early onset of cancer, there are significant amount of patients who are at reproductive age have to face infertility tissue caused by their cancer treatment. While most results of oocyte cryopreservation have been obtained from ovarian stimulation protocols for IVF, ovarian stimulation with gonadotropins may not be suitable for many women who need to preserve their fertility, especially those with hormone-dependent cancers, or those require immediate chemotherapy. While IVM of immature oocytes after frozen-thawing remains to be a challenge, for most species, cryopreservation of mature oocytes is agreed to be more effective than freezing immature oocytes (Cao and Chian, 2009). Hence, immature oocyte retrieval followed by IVM and vitrification could be a promising strategy to preserve fertility. Although several studies revealed no differences in oocyte survival rates, integrity of meiotic spindle, and chromosome alignment between in vivo and in vitro matured oocytes followed vitrification-thawing (Huang et al., 2008, Chang et al., 2008). Clinical studies indicated that in vitro matured oocytes were more sensitive to freezing injuries and lower survival rates were obtained after vitrification-thawing when compared to in vivo matured oocytes (Chian et al., 2009, Chian et al., 2008). One of the aspects among multiple factors that may contribute to the suboptimal outcome of IVM oocytes is the oxidative stress that is exerted to the oocytes during in vitro culture (Combelles et al., 2009).

ROSs are formed as intermediary products of cellular metabolism. With its oxidative propriety, they could cause structural and functional alternation of the cell, and involved

in promoting apoptosis pathways. The cell is under oxidative stress whenever the balance between pro-oxidant and antioxidant is disturbed, which may result from an increase of superoxides and free radicals, or a decrease of antioxidant. In both cases, the cell's defense mechanism could no longer cope with the oxidative species formed. During IVM, oocytes are extremely vulnerable to oxidative stress due to the generation of ROS not only from cellular metabolism, but also external influences, such as light, rapid change of oxygen concentration, and the significant amount of in vitro handling.

GSH is a major non-protein sulfhydryl compound in mammalian cells and is known to participate in various mechanisms, such as microtubule assembly (Deneke and Fanburg, 1989), sperm decondensation and male pronucleus formation in different species (Funahashi et al., 1999, Yoshida et al., 1993, Sutovsky and Schatten, 1997), and most importantly, protecting cells from oxidative damage by acting as reactive oxygen species scavengers (Meister, 1983). It has been considered served as a natural reservoir of reducing power and used extensively as a biomarker of OS by the ratio between its oxidized form and reduced form. During maturation and development of the oocytes in the ovary, GSH content increases as the oocytes approach the time of ovulation. GSH concentration in ovulated MII oocytes is approximately the twice as high as in GV stage oocytes in hamster oocytes (Zuelke et al., 2003). After ovulation, early embryonic development is accompanied by a massive oxidation of GSH, with a 55% decrease at the time of zygotic genome activation, and approximately 10-fold decrease during pre-implantation development, from unfertilized oocytes to blastocyst (Gardiner and Reed, 1994, Luberda, 2005). Concomitant to the rapid decrease of GSH, NADPH level also

drops around 45%, which results a shift of intracellular redox state to oxidation (Dumollard et al., 2007). Hence, the accumulation of GSH in the oocytes during development is essential for protecting it in later stages of fertilization (Telford et al., 1990).

Low-molecular weight thiol compounds, such as cysteamine has been frequently supplemented into the medium to increase antioxidative capacity of embryo via raising intracellular levels of GSH (Bannai, 1984, Issels et al., 1988). It has been demonstrated that cysteamine supplementation in IVM medium of mouse oocytes increased the intracellular GSH content (de Matos et al., 2003), and subsequently improved the in vitro blastocyst development (de Matos et al., 2003). This beneficial effect of cysteamine supplementation during IVM was also reported in bovine (Balasubramanian and Rho, 2007, de Matos et al., 1995, de Matos et al., 1996, de Matos et al., 2002a, de Matos et al., 1997), goat (Urdaneta et al., 2004), buffalo (Gasparrini et al., 2000), and pig (Gruppen et al., 1995) models. Furthermore, when GSH synthesis was stimulated by cysteamine during IVM, more embryos developed faster and reached the blastocysts stage at day 6 of culture (de Matos et al., 1996), which are proven to be more developmentally competent and viable than those slower developing ones (Bavister, 1995).

It has been shown that free radicals were formed during cryopreservation of tissues (Whiteley et al., 1992), which might be resulted from the change of temperature and osmotic damage of the cells (Thomson et al., 2009). Microarray analysis also showed that freezing and thawing induced expression of oxidative stress scavengers genes in

eukaryotic cells, which indicates the activation of cellular oxidative defense systems in response of damage produced during vitrification (Odani et al., 2003, Tatone et al., 2010). In addition to the increase of pro-oxidants, the cell's antioxidative defense system might also have been disturbed during the process cryopreservation, such as the damage of antioxidant enzyme and antioxidant gene transcription machineries, altering the antioxidant potential of oocytes after thawing. It has been showed that supplementation of cryopreservation medium with antioxidant enzymes superoxide dismutase (SOD), catalase, nitric oxide scavenger could significantly increase the survival and fertilization capabilities of oocytes, by preventing membrane lipid peroxidation (Dinara et al., 2001). Mouse embryos cryopreserved at the two-cell stage have elevated levels of intracellular H_2O_2 , which was known to induce developmental blockage of mouse embryos (Ahn et al., 2002). Analysis of GSH level in vitrified porcine oocytes revealed that the intracellular level of GSH in vitrified oocytes was lower than in the fresh oocytes, which suggested cooling and vitrification induces GSH loss in oocytes which might cause them more vulnerable to oxidative damage (Somfai et al., 2007).

Cytoplasmic defects area big concerns in IVM oocytes, which contributes to low embryonic development competency. In particular, the organization and activity of mitochondria are necessities for cytoplasmic maturation due to its role in energy production. However, Mitochondria is also the richest source of ROS, and extremely prone to oxidative damage -- about 1-2% of oxygen metabolized by mitochondria is converted to superoxide (Balaban et al., 2005).

Mitochondria genome is maternally inherited. It replicates throughout oocytes growth and stop replication in the early cleavage stages of embryogenesis until blastocyst stage in mice (Piko and Taylor, 1987, Ebert et al., 1988), which means the mtDNA has to be amplified to a sufficient level prior to fertilization. Significantly lower mtDNA copy numbers has been found in the oocytes of the patients who has repeated fertilization failure (Reynier et al., 2001). It has been proposed that a critical threshold of 100 000 copies in MII stage oocytes for them to be competent in subsequent embryonic development (Piko and Taylor, 1987). During oocytes maturation, mtDNA replication and the expression of the relevant transcription and replication factors, such as POLG (polymerase gamma) and TFAM (mitochondrial transcription factor A) are highly regulated in the porcine oocytes that are likely to be fertilize and develop (Spikings et al., 2007). However, little is known about the impact of in vitro culture on the regulation of mtDNA replication. There is only one study showed that the variation of mtDNA copy number from fertilization to birth was associated with oxidative stress (Aiken, 2008).

The present study was undertaken to look at the effect of cysteamine supplementation during IVM on the early embryonic development after vitrification and IVF. In the second part, we investigated one of the possible underlying mechanism which is whether the change of oxidative status could affect the mtDNA replication during IVM, and in turn benefits the competency of the oocytes.

3.2 Material and Method

All chemicals and reagents used were purchased from Sigma Chemical Company unless

otherwise stated.

Animals:

In all experiments, CD1 mice were used (female age of 8-10 weeks; male age of 10-12 weeks). Mice were housed in environmentally controlled room under a 12L:12D photoperiod, with free access to food and water. The experimental protocols and animal handling procedures were reviewed and approved by Animal Ethics Committee of McGill University.

Collection of Oocytes:

For mature oocyte (MII) collection, female mice were superovulated by intraperitoneal injection of 10IU Pregnant Mare Serum Gonadotrophin (PMSG), followed by Human Chronic Gonadotrophin (HCG) for induction of ovulation after 48 hours of PMSG. After 14-16 hour of HCG injection mice were sacrificed by cervical dislocation. Oviducts were dissected into a petri dish containing washing medium (mHTF-HEPEs supplemented with 1.0 mg/ml BSA). COCs were released by tearing the ampullae of the oviducts.

For immature oocytes (GV) collection, mice were sacrificed 44-48 hrs after PMSG injection. Ovaries were collected into the washing medium, and COCs were collected by puncturing visible follicles using a 25-gauge needle. Deduced GV stage oocytes were obtained by mechanically pipetting COC repeated through a fine glass pipette

In Vitro Maturation of the Oocytes:

Both COCs and denuded oocytes were incubated in Oocyte Maturation Medium (protocol

refer to appendix I) supplemented with 75 mIU/ml recombinant human FSH and LH (Ferring Inc, Ontario, Canada), 10% FBS, with the presence or absence of cysteamine (200 μ M). 8-10 COCs were cultured per 50 μ L medium drop, in a 35mm culture dish (Falcon), covered with mineral oil. Oocytes were kept in the incubator at 37.0°C with an atmosphere of 5% CO₂ and 95% air and high humidity. After 16 hours of culture, COCs were denuded from cumulus cells by repeated pipetting with a fine glass pipette in mHTF-HEPEs (protocol refer to appendix III) supplemented with 100IU/ml hyaluronidase for assessment of their maturity. Matured oocytes were determined by the extrusion of their first polar body.

Vitrification and Thawing:

Based on the method previously described by Huang et al., (Huang et al., 2008) oocytes were suspended in mHTF-based equilibration medium (20% FBS, 7.5% EG, 7.5% PROH) for 3 min and transferred to vitrification medium (20% FBS, 15% EG, 15% PROH, and 0.5M sucrose) for 45-60 sec at room temperature. Normally around 5-10 oocytes were loaded onto a vitrification device called McGill Cryoleaf (MediCult Company, Denmark) and immediately plunged into LN₂ for storage.

For thawing, the Cryoleaf was directly inserted into the 37°C thawing medium (20% FBS, 1.0M sucrose) for around 1min, then to the dilution medium 1 (0.5M sucrose) and 2 (0.25M sucrose) for 3 min respectively. Thawed oocytes were washed twice in mHTF-HEPEs medium for 3 minutes before being transferred to the fertilization medium for IVF.

Sperm Capacitation and Fertilization:

Sperms were directly squeezed from the cauda epididymides of male mice to a capacitation droplet (40µl) covered with mineral oil. Capacitation medium was based on mHTF (protocol refer to appendix II) and supplemented with 9 mg/ml BSA. The time for sperm capacitation was 60 – 90 min. Cumulus free IVM and in vivo matured MII oocytes were inseminated with capacitated spermatozoa at a concentration between $1-25 \times 10^6$ sperm/ml. Gametes were co-incubated for 6 hrs, and zygotes were transferred to the embryo maintenance medium (Cooper Surgical/SAGE, Lawrence, KS, USA) for embryonic development.

Assessment of Fragmentation:

Fragmentation rates of embryos were observed at Day 2 of culture when most of the oocytes were at 2-cell stages. Cleaved embryos with all level of fragmentation visible in the cytoplasm were counted as fragmented.

DNA Extraction:

DNA extraction was performed using DNA extraction kit, QiAamp DNA Micro (Qiagen) under the protocol of *Isolation of Genomic DNA from Small Volumes of Blood*. 17 oocytes were grouped per sample for DNA extraction. Rabbit Globin cDNA was added to the sample as external control prior to oocyte DNA extraction. Samples were diluted in 1:10 with sterile ddH₂O before PCR analysis.

Preparation of external standard for Real-time PCR:

The mixture of 10µl rabbit globin mRNA(15pg), 1µl of 20uM random hexamers and 1µl of 10mM dNTP was incubated at 65°C for 5min and rapidly chilled on ice prior to reverse transcription(RT). RT was performed at 42°C for 50min in a final volume of 20µl containing 4µl of 5 X RT buffer, 2µl of 0.1M dithiothreitol, 0.5µl RNase-free water and 0.5µl (100U) of superscript II reverse transcriptase (Invitrogen) followed by incubation at 70 °C for 10min to inactivate the RT reaction. The cDNA product was 2 times diluted with sterile water and conserved in -20°C in single-use aliquots.

Real-time PCR for mt DNA copy number relative quantification:

Real-time PCR was performed on the Corbett Rotorgene 6000 cyclor (Qiagen), with a 36 well rotor. mtDNA level was analyzed with QuantiTect SYBR Green PCR master mix (Qiagen) in a total reaction volume of 20 µl , containing 0.25µM primer, 10µl of SYBR dye, 7µl of sterile ddH₂O, and 2µl of cDNA sample. Oligonucleotide primers used for the amplification of the target sequences of mtDNA were synthesized by Sigma, Inc. The nucleotide sequence and sizes were shown in TABLE 3.1 PCR reaction conditions were 95 °C for 15 min, followed by 50 cycles consisting of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 35 s. Quantification was normalized with the external control (Rabbit Globin cDNA). The relative levels of mtDNA per fixed number of oocytes were calculated by the Rotorgene software, using Comparative Quantification method (Qiagen) and Microsoft Excel.

TABLE 1 : Primers used for real-time PCR

Gene	Forward primer	Reverse primer	Annealing temp(°C)	Extension time(sec)	Primer Concentration (µM)	Product size(bp)
Mt DNA-ND1	CGA TTA AAG TCC TAC GTG ATC TGA	CTG GGA GAA ATC GTA AAT AGA TAG AAA	60	35	0.25	80bp
Rabbit Globin cDNA	GTG GGA CAG GAG CTT GAA AT	GCA GCC ACG GTG GCG AGT AT	60	35	0.25	257bp

Experimental Design:

The first part: Both denuded and cumulus intact GV stage oocytes were matured in the presence or absence of cysteamine for 16 hours. Matured oocytes were either inseminated directly through IVF or subjected to vitrification, stored in the liquid nitrogen for at least 1 week, and followed by IVF after thawing. In all groups, maturation rate, cleavage rate and fragmentation rate were assessed.

The second part: The relative levels of mtDNA copy number of oocytes were assessed using Real-time PCR. The relative mtDNA copy numbers of GV and MII stage oocytes were first analyzed. For GV and MII stage oocyte mtDNA comparison, two separate samplings were done, and 17 oocytes per each sampling were collected. For the effect of supplementation of cysteamine, oocytes matured in three different conditions (in vivo matured and in vitro matured oocytes with or without the supplementation of cysteamine) were analyzed. Three separate samplings were done, and 3 pools (17 oocytes per pool) for each condition per sampling were carried out in parallel for DNA extraction and Real-time PCR analysis, which made up to 9 separate DNA samples per conditions.

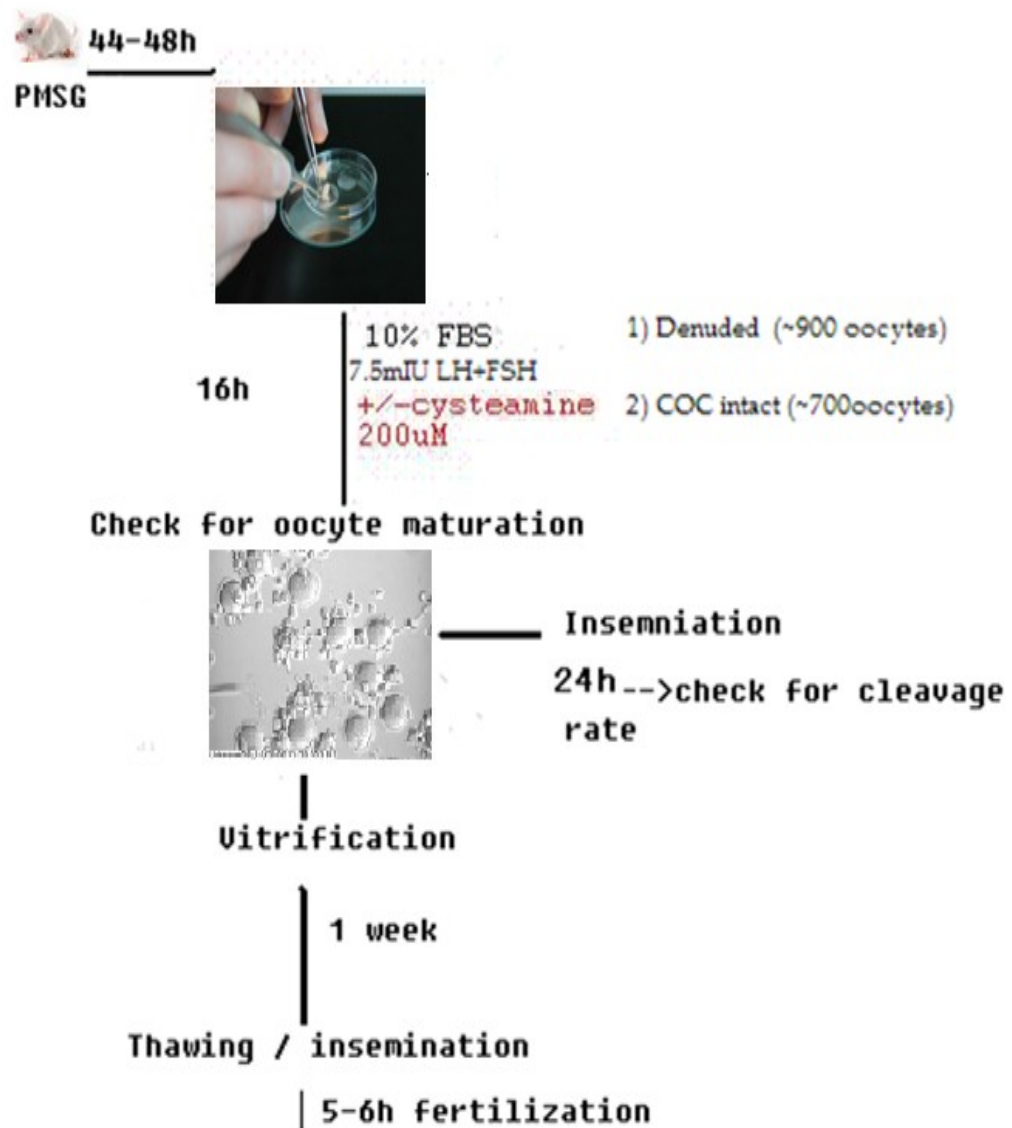


Fig 3.1 Experimental Procedure

Statistical Analysis:

One-way ANOVA was used to compare the means of maturation, cleavage, fragmentation rate for each condition. The chi-square test was used to analysis the differences in the case of percentage comparison, such as ratio of mtDNA copy number. Significant difference was defined as $P < 0.05$.

3.3 Results

In terms of fragmentation rate in unvitrified groups, there was no significant difference in oocytes matured with or without cysteamine supplementation in both denuded and COC groups. (Table 3.2) Comparing COC groups with denuded groups in unvitrified condition, without the supplementation of cysteamine, fragmentation in denuded group was significant higher ($P < 0.05$) than in COC groups, however with the presence of cysteamine, there were no such significant difference observed between denuded and COC groups.(Table 3.3, 3.4)

In vitrified groups, with the addition of cysteamine, fragmentation rate was significant lower ($P < 0.05$) than un-supplemented groups in COC groups, and brought it to a level that was similar to their corresponding unvitrified groups. However, even with the addition of cysteamine, the fragmentation levels were similar in both denuded groups. Fragmentation rates were generally higher in vitrified groups than in unvitrified groups (Table3.4).

There was no significant difference in the maturation rate and cleavage rates between groups matured with or without the supplementation of cysteamine (200 μ M), in both COC and denuded groups. Development in terms of 4-8 cell rate and blastocyst rate, were not significantly different between cysteamine treated and untreated groups. However, denuded oocytes have generally significantly lower 4-8 cell rate than COC intact groups (Table 3.5, 3.6).

Fluorescent real-time quantitative analysis of mtDNA in GV and MII oocytes showed that the ratio of mtDNA copy number of MII oocytes were 1.4 times of GV stage oocytes amplified by mtDNA primer ND1(Fig 3.4), which agrees with the previous publications that immature mouse oocytes have a lower mtDNA copy number than MII stage oocytes (Piko and Taylor, 1987). However, in this study the difference was not statistically significant.

For the second part, we compared the mtDNA copy number of the three groups, in vivo matured oocytes, in vitro matured oocytes with or without the supplementation of cysteamine. The ratio of mtDNA copy number of IVM oocytes to in vivo matured oocytes was 0.89, with no statistically significant difference. mtDNA copy numbers were similar in cysteamine treated and untreated groups, with a ratio close to 1. (Fig 3.5) Interestingly, the variance of mtDNA copy number between the 9 samplings of in vivo matured oocytes was around 2-4 times greater than in vitro matured groups. (Fig3.5)

Table 3.2. Maturation rates of cumulus-intact and -free GV stage oocytes with or without the supplementation of Cysteamine during IVM (9 replicates)*

Treatment	No. of oocytes examined	No. of oocytes Matured (%)
COC + Cys	466	422 (90.6)
COC - Cys	418	358 (85.6)
Denu + Cys	348	294 (84.5)
Denu - Cys	340	280 (82.4)

*** There were no significant differences among groups.**

Table 3.3. Effect of the supplementation of Cysteamine in IVM-medium during IVM on early embryonic fragmentation without vitrification (6 replicates)*

Treatment	No. of 2-cell embryo	No. of fragmented embryos (%)
COC + Cys	127	52 (40.94)a
COC - Cys	111	37 (33.33)a
Denu + Cys	61	31 (50.82)ab
Denu - Cys	101	64 (63.37)b
Control	65	3 (4.6)c

* Within column, values with different superscripts are significantly different (P<0.05).

Table 3.4. Effect of the supplementation of Cysteamine in IVM-medium during IVM on early embryonic fragmentation following vitrification (6 replicates)*

Treatment	No. of 2-cell embryos	No. of fragmented embryos (%)
COC + Cys	125	51 (40.8)a
COC - Cys	134	83 (61.9)b
Denu + Cys	98	73 (74.5)b
Denu - Cys	77	54 (70.1)b
Control	65	3 (4.6)c

* Within column, values with different superscripts are significantly different (P<0.05).

Table 3.5. Effect of the supplementation of Cysteamine in IVM-medium during IVM on early embryonic development (6 replicates)*

Treatment	No. of 2-cell embryos	No. of 4-8 cell stage embryos (%)	No. of blastocyst (%)
COC + Cys	127	52 (40.9)a	3 (2.4)a
COC - Cys	111	45 (40.5)a	1 (0.9)a
Denu + Cys	61	13 (21.3)b	0 (0.0)a
Denu - Cys	101	18 (17.8)b	2 (2.0)a
Control	65	57 (87.7)c	57 (87.7)b

* Within column, values with different superscripts are significantly different (P<0.05).

Table 3.6. Effect of the supplementation of Cysteamine in IVM-medium during IVM on early embryonic development of vitrified oocytes (6 replicates)*

Treatment	No. of 2-cell embryos	No. of 4-8 cell stage embryos (%)	No. of blastocysts (%)
COC + Cys	204	23 (18.4)bc	0 a
COC - Cys	183	38 (28.4)ab	0 a
Denu + Cys	131	10 (10.2)c	0 a
Denu - Cys	99	6(7.8)c	0 a
Control	83	57 (87.7)e	57 (87.7)b

* Within column, values with different superscripts are significantly different (P<0.05).



Fig 3.2. 2-cell stage embryos with different levels of fragmentation

a: 2-cell stage embryo with minor fragmentation. Both blastomeres were intact. b: 2-cell stage embryo with one blastomere partially fragmented. c: 2-cell stage embryo with one blastomere completely fragmented.

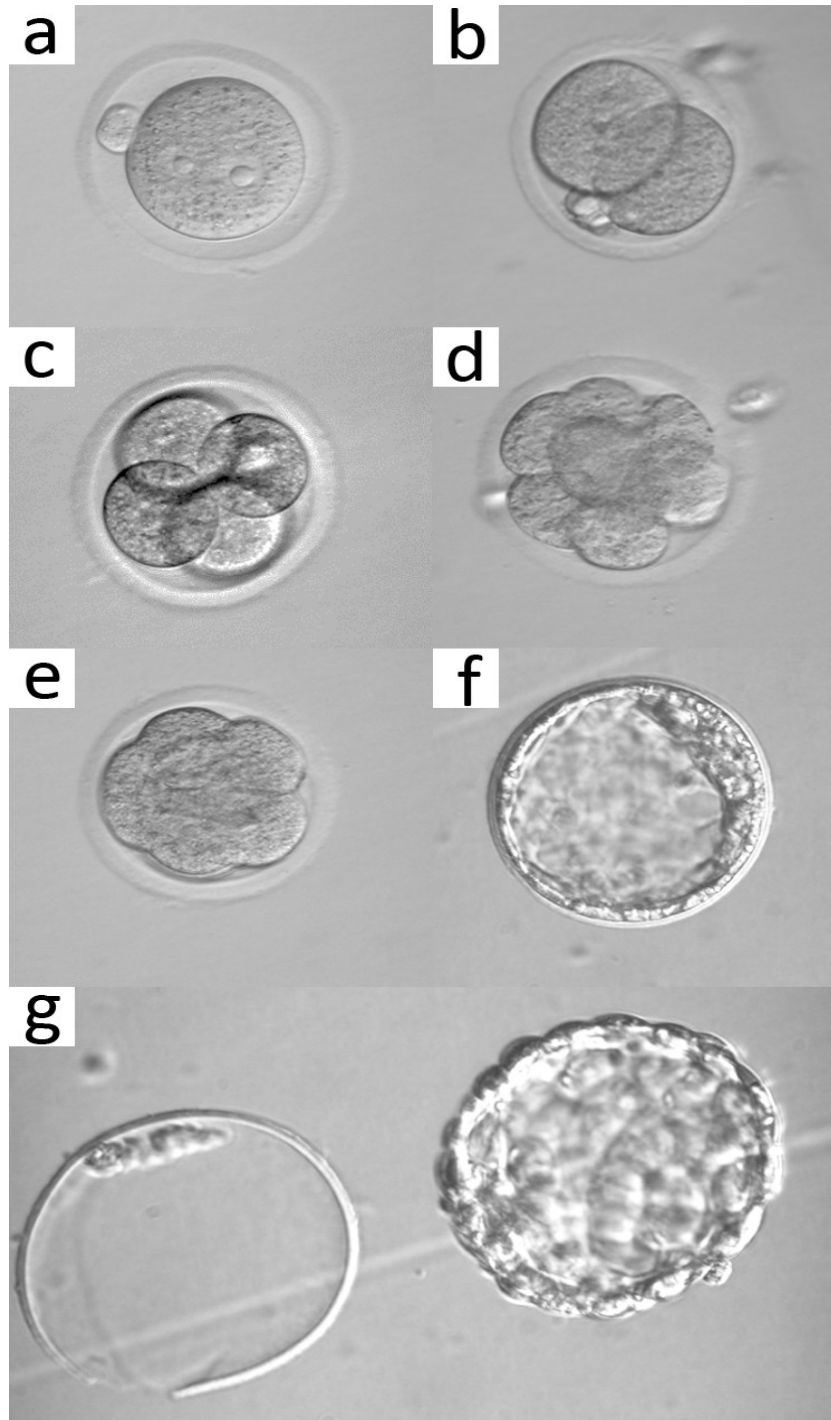


Fig 3.3 Early embryonic development after IVF

a: Fertilized oocytes with two pronucleus visible. b: 2-cell stage embryo c: 4-cell stage embryo d: 8-cell stage embryo e: Morula stage embryo f: Blastocyst stage embryo g: hatched blastocyst

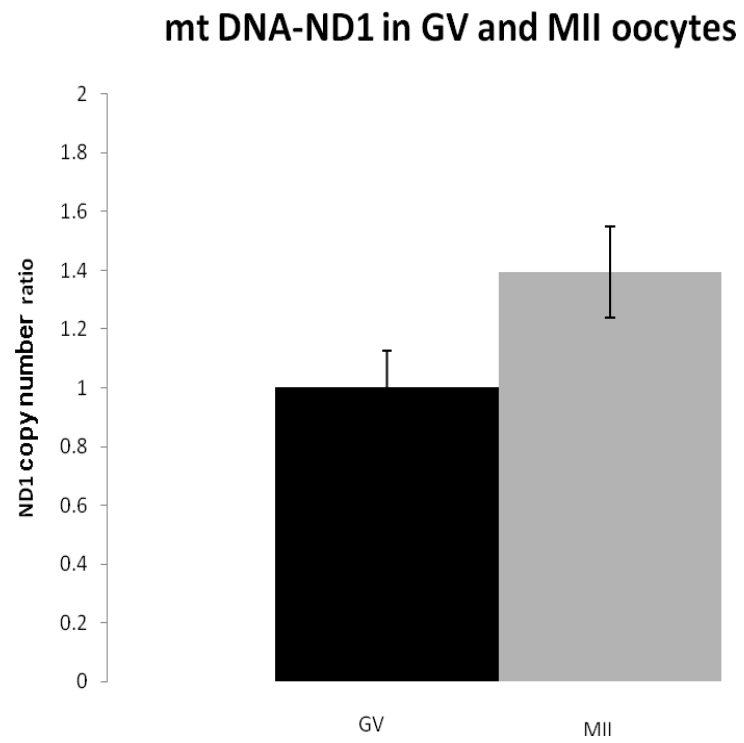


Fig 3.4.: The relative level of mtDNA-ND1 , copy ratio between GV and MII oocytes.

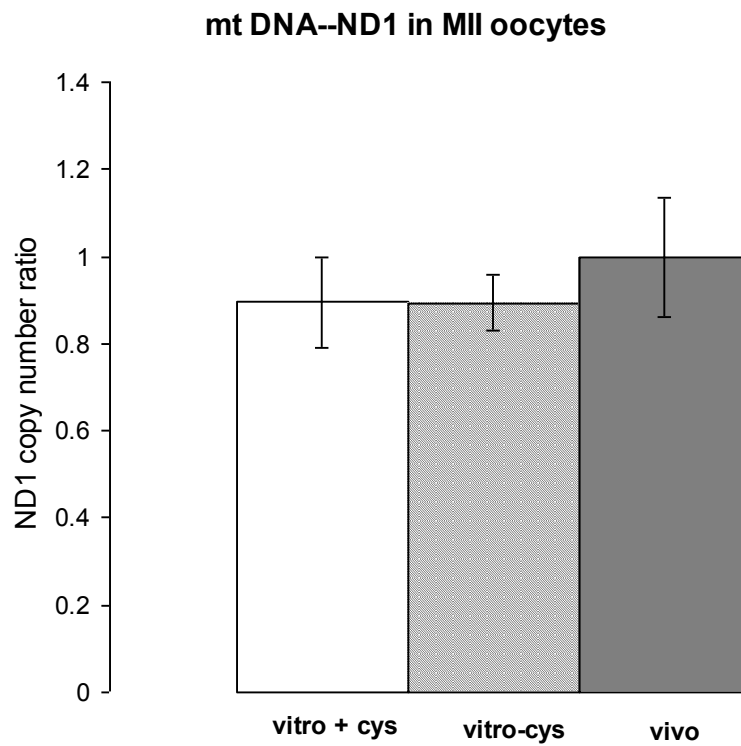


Fig 3.5: The relative level of mtDNA-ND1 copy ratio between in vivo matured, in vitro matured with or without cysteamine supplementation during IVM.

3.4 **Discussion**

Although the mechanism of cysteamine function is not completely understood, the main protective role of cysteamine to the cell is to prevent the oxidation of cysteine, a precursor of GSH, and to facilitate its entry into the cell, resulting an increased GSH synthesis (Deleuze and Goudet, 2010). De novo GSH synthesis during maturation of mouse oocytes is essential requirement for oocytes competence, and it serves as a reservoir to protect the oocytes and zygotes from oxidative damage (Luberda, 2005). The inhibition of GSH synthesis during IVM has been reported to result a decreased embryonic development (de Matos et al., 2003).

Studies in many species, bovine (de Matos and Furnus, 2000, de Matos et al., 1995, Anand et al., 2008), mouse (de Matos et al., 2003), equine (Luciano et al., 2006), and Canine (Hosseini et al., 2007) have shown that the supplementation of cysteamine during oocytes maturation and embryonic culture could facilitate the embryonic development in a concentration ranging from 50 μ M to 500 μ M. However, the effect of one given concentration were reported differently between studies. Supplementation of IVM medium with 500 μ M (Gruppen et al., 1995), 100 μ M /200 μ M (Urdaneta et al., 2003), 100 μ M/200 μ M (de Matos et al., 1995) cysteamine in porcine, goat , and bovine oocytes respective, resulted improved developmental capacity of the oocytes. This discrepancy could partially be explained by the different culture environments and mediums used for each study.

Reviewing the publications, some have reported the beneficial effect of the supplementation of cysteamine in IVM medium on the nuclear maturation rate (Hossein et al., 2007, Chen et al., 2005, Urdaneta et al., 2003, Bing et al., 2001), while others, even with the same animal species, reported no significant increase in nuclear maturation (Zhou et al., 2008, Yamauchi and Nagai, 1999, Luciano et al., 2006, Oyamada and Fukui, 2004). In our study, the nuclear maturation rates were slightly higher upon cysteamine supplementation in both COC and denude groups, however the difference was not significant (Table 3.2). The denuded group showed a slightly lower nuclear maturation rate comparing to COC group, with also no significant difference (Table 3.2). Our results suggested that nuclear maturation itself may not be sensitive to the change of redox stage of the oocytes in the current culture environment. On the other hand, the discrepancy between various studies may be resulted from the different culture environments. For those studies which had significant increase in nuclear maturation rate, it could be partially explained by the excess of oxidative stress in their culture systems. The increase of GSH synthesis helped to rescue ROS damage, and when the cysteamine was not supplemented, the oxidative stress was at a level that affected the nuclear maturation of the oocytes.

In consistent with the suggestion that supplementation of cysteamine could increase the resistance of the embryo to oxidative stress, which in turn reduce DNA-fragmentation (Hosseini et al., 2009, Feugang et al., 2004), we have found that there was a significant decrease in DNA fragmentation at Day 2 in cysteamine supplemented groups of vitrified oocytes (Table 3.4). Embryos are extremely sensitive to oxidative stress at 2-cell stage,

during the maternal to zygotic transition. It is usually observed with an increase of ROS accumulation (Guerin et al., 2001, Nasr-Esfahani and Johnson, 1992). This is likely to be caused by the depletion of oxidative defense at the critical window of zygotic genome activation (Dumollard et al., 2009). After zygotic genome activation, the increased oxidative stress will usually trigger fragmentation and apoptosis of the embryo (Dumollard et al., 2009).

Vitrified oocytes generally have increased fragmentation after fertilization comparing to the fresh ones. The higher fragmentation rate in oocytes that undergo vitrification may be related to the excess of oxidative stress and the toxic effect of cryoprotectants during vitrification (Huang et al., 2008). A beneficial effect of cysteamine supplementation on vitrified embryos, both before vitrification and during vitrification have been reported by Hosseini et al. (Hosseini et al., 2009).

In our study, we found that the effect of cysteamine on the decreasing fragmentation at 2-4cell stage was only observed in COC group, but not denuded groups (Table 3.4). It showed that without the cumulus oocytes interaction, the oocytes' ability to uptake GSH was not efficient, which suggested the facilitated roles of cumulus in the GSH synthesis in the oocytes during oocytes maturation. The similar result was also reported in porcine oocytes. GSH content in matured oocytes were significant higher in COC group than denuded group (Maedomari et al., 2007, Bing et al., 2002).

Despite the considerable amount of studies in the effect of cysteamine supplementation during oocyte IVM, seldom had looked into its underlying mechanism. This cysteamine exerted effect during IVM could be long term that benefits the later development or short term through altering of the property of the oocytes during IVM. The long term effect might be explained by the pre-enriched GSH which stored in the oocytes, and used to reinforce oxidative defense during vitrification or at early development stage when it is under the oxidative stress. However, it has been suggested that the supplementation of antioxidant at the time of ROS production was more effective than pre-enrichment on changing the embryos redox state (Hosseini et al., 2009). The short term effect could be explained by the effect of cysteamine at the time of maturation on the cytoplasmic maturation, which strength the oocytes ability for their later activities.

In this study, we tested the hypothesis of the short term effect that the change of redox state during IVM on the regulation of mtDNA replication during oocyte maturation. In mice embryos, mitochondria do not resume replication until blastocyst stage hence maternal mitochondria must be distributed to all resulting blastomeres. It has been proposed that oocytes need to reach a critical threshold of mtDNA copy number at the time of maturation in order to be competent in the fertilization and early embryonic development since a sufficient ATP production must be ensured for subsequent embryonic activities (Van Blerkom et al., 1995). In other word, mitochondrial biogenesis may be an important indicator for the level of cytoplasmic maturation of the oocytes.

Reviewing previous publication on mtDNA quantification, there was a big variation in the reported mtDNA copy number. The early quantification with dot blot analysis method revealed copy number of 119,000 in mouse oocytes (Piko and Taylor, 1987), and more recent PCR results reported a copy number of 157,000 in mouse (Cao et al., 2007). With Real time PCR method, Steuerwald et al. reported a copy number of 314,000 (Steuerwald et al., 2000); Reynier et al. showed a copy number of 209,000 ((Reynier et al., 2001), and Chen et al reported a copy number of 138,000 in single human oocyte. Not only among studies, big mtDNA variation was also seen between samples in the same study. For example, Steuerwald et al. reported an average mtDNA copy number of 314,000, with a range of concentrations among their samples from a low of 90,000 to a high of 600,000 (Steuerwald et al., 2000). Hence, the difference in the mean mtDNA content of oocytes was resulted between both the large inter-individual variations, as well as the oocytes belonging to a given cohort.

By the semi-quantitative quantification method used in this study, we compared the ratio of mtDNA amount in the GV and MII stage oocytes, In vivo and In vitro matured oocytes, and those matured with or without the presence of cysteamine. We found that the ratio of mtDNA copy number of MII oocytes were 1.4 time of GV stage oocytes according to two samplings (Fig 3.4), which agrees with the previous publications that immature mouse oocytes have a lower mtDNA copy number than mature oocytes (Piko and Taylor, 1987). However, the difference was not significant statistically. The results suggested that there may be an increase in mitochondrial content during the final stage of oocytes maturation,

which contributes to the oocytes cytoplasmic maturation.

In vitro matured oocytes have a lower mean copy number than in vivo matured oocytes with a ratio of 0.89, but with no statistical significant difference. Interestingly, mtDNA copy number of in vivo matured oocytes showed a 2 to 4 time greater variation than the two in vitro matured groups (with or without cysteamine supplementation during IVM) (Fig 3.5). It may suggest that oocytes ovulated at MII stage after hCG induction may be potentially different in cytoplasmic maturity, which leads to cohorts of oocytes at various maturation stages. This phenomena was less obvious in the in vitro matured oocytes, when oocytes were manually selected based on their size and compactness of cumulus cells from the big follicles, without hCG stimulation. However, this assumption is needed to be validated by future studies.

mtDNA copy number were consistent between cysteamine treated and untreated groups, with a ratio close to 1. This result suggested that the beneficial effect of cysteamine supplementation during IVM was not facilitated through the change of mtDNA content during oocytes maturation. The increase of antioxidant defense did not directly benefit to the mtDNA accumulation during IVM.

In conclusion, we have demonstrated that the addition of cysteamine (200 μ M) during oocyte IVM has significantly reduced fragmentation of vitrified oocytes in the context of intact cumulus cells. The presence of cumulus cells was necessary for this beneficial

effect. The functional role of cysteamine on oocyte maturation in vitro may not be directly related to mtDNA replication.

Chapter 4: Experiment 2

Disturbance of mitochondrial function during oocyte maturation on the survival and embryonic development following vitrification and IVF

Summary:

The purpose of this study is to disturb the mitochondria function using a specific mitochondria functional blocker during oocyte IVM, and to investigate the correlation in induced mitochondria dysfunction to the vitrification outcome of MII oocytes. Testing of rotenone concentration from 100nM to 50 μ M, showed a lethal dose around 50 μ M when all the oocytes were lysed during IVM, and a dose related significant increase of death from 10 μ M to 50 μ M. With the increase of rotenone concentration from 2 μ M to 50 μ M, the maturation rates were dropped abruptly in a dose dependent manner to 0 eventually when the concentration reached 50 μ M. Within the concentration window of 250 nM to 2 μ M, where no difference have been found in terms of the death rate and maturation rate comparing to their relative control, there is a significant drop of cryo-survival rate at a concentration of 2 μ M (from 93.3% to 55.6%). The number of embryos reached to 4-8 cell stage were significant lower at 2 μ M group (17.5%), comparing to lower concentration, 250 nM (50%). In conclusion, disturbance of mitochondria function during oocyte maturation to a certain level, where no difference was seen in terms of maturation outcome, could still significantly affect cryo-survival and early embryonic development after vitrification and IVF. It confirms the strong positive correlation of mitochondrial

functionality during oocyte maturation on its later cryopreservation and embryonic development outcomes.

4.1 Introduction

Oocyte quality is negatively affected by the maternal age, which results poor fertilization, embryonic development and cryopreservation outcomes. The risk of trisomy pregnancy is around 2-3% in women among the age of early 20s, however, the risk can increase to 30-35% in women at their 40s (Hassold and Chiu, 1985). The age related error can happen at both meiosis I and meiosis II, as a result of predivision or nondisjunction of chromatid (Plachot, 2003, Bugge et al., 1998, Yoon et al., 1996). An increase in aneuploidy is believed to be the major underlying factor that is responsible for the compromised oocyte quality in women with advanced age (Pellestor et al., 2002, Hassold and Hunt, 2001, Pan et al., 2008).

The correct segregation of meiotic chromosome/chromatid depends on the dynamics of spindle. Both the synapsed chromosome pairs in MI and sister chromatid in MII have to be pulled to the opposite ends of the dividing oocytes which is guided by the extending and shortening of the meiotic spindles (Eichenlaub-Ritter et al., 1996). The function of the mitotic spindle appears to be highly dependent on ATP (Van Blerkom, 1991, Takeuchi et al., 2005). In the oocytes, majority of the ATP are produced by mitochondria, which are the most abundant organelle in the oocytes. Mitochondria are distributed in particular patterns during the process of maturation, and early embryonic development to provide

fuel for critical cellular events. During GV stage, mitochondria are homogenous distributed in the cytoplasm, and at the time of GVBD to MII, mitochondria aggregate around the nucleus (Van Blerkom and Runner, 1984, Dell'Aquila et al., 2009).

Mitochondria are extremely prone to oxidative damage, as a higher concentration of oxygen species presented in the mitochondria due to its role in energy production (Richter et al., 1998). Oocytes from older women showed an accumulation of mitochondria point mutation, deletions, which results in respiratory chain defects and leading to a compromised ATP production (Shigenaga et al., 1994, Barritt et al., 2000). It has been shown that the transfer of ooplasm from younger to older eggs benefits in rejuvenating fertility in older women, and to treat the age-related cytoplasmic dysfunction responsible for oocyte aneuploidy (Zhang et al., 1999, Takeuchi et al., 2001). Hence, the age-related increase in mtDNA mutations could be an underlying cause of chromosomal non-disjunction caused by the advanced maternal age. (Van Blerkom et al., 1998, Schon et al., 2000).

Rotenone is a specific mitochondria blocker that interferes with the transfer of electrons from complex I to ubiquinone on the mitochondria electron transport chain. This results a compromised mitochondria electric transport chain function and hindered ATP production. Study has shown that rotenone delayed cell progression in all phases of the cell cycle of mammalian cell mitosis by direct respiration inhibition, and as one of the consequence, it inhibits spindle microtubule assembly (Barham and Brinkley, 1976). Since there hasn't

been a lot of available information on the effect of rotenone on mouse oocyte, a dose finding is performed to find out a dose window of the viability and the maturation of oocytes.

The purpose of this study is to disturb the mitochondria function using a specific mitochondria functional blocker during oocyte IVM, and to investigate the dose window that could correlates the level of mitochondria function blockage to the vitrification outcome. We hypothesized that since mtDNA mutations accumulates as women ages, the aneuploidy will increase as a result of increasing in severity of mitochondria dysfunction, which affects the oocytes recovery after freezing and warming.

4.2 Material and Methods:

All chemicals and reagents used were purchased from Sigma Chemical Company unless otherwise stated.

Animals:

In all experiments, CD1 mice were used (female age of 8-10 weeks; male age of 10-12weeks). Mice were housed in environmentally controlled room under a 12L:12D photoperiod, with free access to food and water. The experimental protocols and animal handling procedures were reviewed and approved by Animal Ethics Committee of McGill University.

Collection of GV stage Oocytes:

Female mice were superovulated by intraperitoneal injection of 10IU Pregnant Mare Serum Gonadotrophin (PMSG), and sacrificed 44-48 hrs after PMSG injection. Ovaries were collected into the washing medium (mHTF-HEPEs supplemented with 1g/ml BSA), and COC were collected by puncturing visible follicles using a 25-gauge needle.

In Vitro Maturation of the Oocytes:

COCs were incubated in Oocyte Maturation Medium (protocol refer to appendix I) supplemented with 75 mIU/ml recombinant human FSH and LH (Ferring Inc, Ontario, Canada), 10% FBS, and corresponding concentration of rotenone. 8-10 COCs were cultured per 50µL medium drop, in a 35mm culture dish (Falcon), covered with mineral oil. Oocytes were kept in the incubator at 37.0°C with an atmosphere of 5% CO₂, 95% air and high humidity. After 16 hours of culture, COCs were denuded from cumulus cells by repeated pipetting with a fine glass pipette in mHTF-HEPEs (protocol refer to appendix III) supplemented with 100IU/ml hyaluronidase for assessment of their maturity. Matured oocytes were determined by the extrusion of the first polar body.

Vitrification and Thawing:

Based on the method previously described by Chian et al., (Huang et al., 2008), oocytes were suspended in mHTF-based equilibration medium (20% FBS, 1.3M EG, 1.0M PROH) for 3 min and transferred to vitrification medium (20% FBS, 2.6 EG, 1.0M PROH, and 0.5M sucrose) for 45-60 sec at room temperature. Normally around 5-10 oocytes were loaded onto a vitrification device called McGill Cryoleaf (MediCult Company, Denmark)

and immediately plunged into LN2 for storage.

For thawing, the Cryoleaf was directly inserted into the 37°C thawing medium (20% FBS, 1.0M sucrose) for around 1min, then to the dilution medium 1 (0.5M sucrose) and 2 (0.25M sucrose) for 3 min respectively. Thawed oocytes were washed twice in mHTF-HEPEs medium for 3 minutes before being transferred to the fertilization medium for IVF.

Sperm Capacitation and Fertilization:

Sperms were directly squeezed from the cauda epididymides of male mice to a capacitation droplet (40 µl) covered with mineral oil. Capacitation medium was based on mHTF(protocol refer to appendix II) and supplemented with 9 mg/ml BSA. The time for sperm capacitation was 60 – 90 min. Cumulus free IVM and in vivo matured MII oocytes were inseminated with capacitated spermatozoa at a sperm concentration between 1-25 x 10⁶ sperm/ml. Gametes were co-incubated for 6 hrs, and zygotes were transferred to the embryo maintenance medium(Cooper Surgical/SAGE, Lawrence, KS, USA) for development.

Rotenone Preparation:

Rotenone was dissolved in DMSO as a stock solution at concentration of 7x10⁻³g/ml. It was diluted in 10 to 100 times with IVM medium and added to the IVM medium according to the corresponding concentration. The preparations of different concentrations of rotenone in 1ml IVM medium are as follow:

Final Rotenone concentration	Dilution of the Rotenone stock solution	Volume of the diluted Rotenone solution
100 nM	500X	2.8µl
250 nM	500X	7µl
500 nM	100X	2.8µl
1 µM	100X	5.6µl
2 µM	100X	11.23µl
4 µM	100X	22.46µl
5 µM	10X	2.8µl
10 µM	10X	5.6µl
25 µM	10X	14.1µl
40 µM	10X	22.5µl
50 µM	10X	28.2µl

Assessment of fragmentation:

Fragmentation rates were observed at Day 2 of culture when most of the oocytes were at 2-cell and 4-cell stages. Cleaved embryos with all level of fragmentation visible in the cytoplasm were counted as fragmented.

Experimental Design:

In the first part of the experiment, we performed a dose finding experiment, looked for a

window of rotenone concentration that could compromise the maturation, and survival of oocytes during IVM. We investigated that whether the developmental potential and fragmentation of IVM oocytes are affected in a dose dependent matter within a known concentration window. Oocytes were matured in IVM medium supplemented with increasing concentration of rotenone. Matured oocytes were subjected to IVF. Early embryonic development and fragmentation rate were recorded. Three repeats were done in each concentration groups.

In the second part of the experiment, we looked at the cryo-survival and early embryonic development of IVM oocytes in groups with rotenone concentrations between 250nM and 2µM, in which range maturation rate was not different from the control group. Oocytes were matured in the maturation medium supplemented with corresponding rotenone concentration. Matured oocytes were vitrified and stored in liquid nitrogen for at least 1 week, followed by thawing and IVF. 6 repeats were done for each rotenone concentration.

Statistical Analysis:

One-way ANOVA was used for the comparison of the means in each condition, such as the maturation rate, fertilization rate, fragmentation rate and cryosurvival rate. Significant difference was defined as $P < 0.05$.

4.3 Results

Effect of rotenone blocked mitochondria function during IVM on the oocyte

maturation, cleavage, and early embryonic development after IVF – a dose finding experiment

Testing of rotenone concentration from 100 nM to 50 μ M, showed a lethal dose around 50 μ M when all the oocytes were lysed during IVM, and significant dose related increase of death was shown from 10 μ M to 50 μ M. There was no trend of lethality when the rotenone concentration was lower than 5 μ M.

The maturation rate wasn't affected when the dose of rotenone was under 2 μ M. With the increase of rotenone concentration from 2 μ M to 50 μ M, the maturation rate was dropped significantly in a dose dependent manner to 0, when the concentration reached 50 μ M. The cleavage rates, and fragmentation rates of matured oocytes were similar among groups (Table 4.1). The blastocyst rates were significantly lower when the concentrations of rotenone were at 5 μ M and 10 μ M (Table 4.1).

Effect of rotenone blocked mitochondria function during IVM on the survival, fertilization and embryonic development after vitrification.

Within the concentration window of 250 nM to 2 μ M, where no difference have been found in terms of the death rate and maturation rate comparing to their relative control, a second part of the experiment has been done to future investigate whether the partial block of mitochondria function affects the survival and embryonic development of oocytes after vitrification.

The cryo-survival rates were significantly lower in the 2 μ M rotenone concentration group than groups with lower rotenone concentration and control (93.3% vs 55.6%) The amount of fertilized embryos reached 4-8 cell stage was significant lower at the concentration of 2 μ M (17.5%), comparing to lower concentration, 250 nM (50%). There was no statistical difference on blastocyst rate among groups, likely due to the little number of blastocyst developed in vitrified groups. No significant difference was found in terms of fertilization and fragmentation among groups. (Table 4.2)

Table 4.1. Maturation, death, and early embryonic development of IVM oocyte without vitrification: (3 replicates)

Rotenone Con	Oocyte No	Death after IVM (%)	Matured (%)	Cleavage	Blastocyst	Frag
100 nM	52	0(0)a	51(98.1)a	32(62.7)a	9(28.1)a	6(18.8)a
250 nM	70	0(0)a	68(97.1)a	58(85.3)a	18(31.0)ab	7(12.1)a
500 nM	70	1(1.43)a	68(97.1)a	59(86.8)a	14(23.7)ab	6(10.2)a
1 μ M	55	0(0)a	53(96.4)a	46(86.8)a	8(17.4)ab	6(13.0)a
2 μ M	53	0(0)a	47(88.7)ab	42(89.4)a	5(11.9)ab	7(16.7)a
4 μ M	54	1(1.85)a	40(74.1)b	31(77.5)a	5(16.1)ab	7(22.6)a
5 μ M	56	1(1.79)a	22(39.3)c	18(81.8)a	0(0)b	5(27.8)a
10 μ M	60	9(15)a	11(18.3)c	11(68.8)a	0(0)b	4(36.4)a
25 μ M	52	26(50)b	2(3.85)d	-	-	-
40 μ M	54	37(68.5)c	1(1.85)d	-	-	-
50 μ M	62	62(100)d	0(0)d	-	-	-
Control	55	0(0)a	54(98.2)a	48(88.9)a	24(50)a	8(16.7)a

* Within column, values with different superscripts are significantly different ($P < 0.05$).

TABLE 4.2. Maturation, death, and early embryonic development of IVM oocyte with vitrification (6 replicates)

Rotenone concentration	250 nM	500 nM	1 μ M	1.5 μ M	2 μ M	Control
Oocyte No	121	135	143	91	153	123
Maturation	121(100) ^a	129(95.6) ^{ab}	137(95.8) ^{ab}	88(96.7) ^{ab}	142(92.8) ^b	117(95.1) ^{ab}
Cyero-survived	113(95.8) ^a	107(87.7) ^a	116(90.6) ^a	83(94.3) ^a	79(55.6) ^b	98(93.3) ^a
Fertilization	74(65.5) ^a	63(58.9) ^a	62(53.4) ^a	46(55.4) ^a	40(50.6) ^a	54(55.1) ^a
Fragmentation	20(27.0) ^a	22(34.9) ^a	20(32.3) ^a	18(39.1) ^a	24(60) ^a	21(38.9) ^a
4-8 cells	37(50) ^a	26(41.3) ^{ab}	15(24.2) ^{ab}	16(34.8) ^{ab}	7(17.5) ^b	29(53.7) ^{ab}
Blastocyst	1(1.35) ^a	4(6.35) ^a	2(3.23) ^a	0(0) ^a	0(0) ^a	3(5.56) ^a

* Within column, values with different superscripts are significantly different (P<0.05).

4.4 **Discussion**

The maturational arrest induced by mitochondria damage was observed in a dose dependent manner as the increase of rotenone concentration from 4 μ M to 50 μ M. Oocytes failed to mature were arrested at GV stage, that couldn't be able to undergo GVBD. GVBD is an energy dependent process; hence the interference of ATP production will compromise this process. The similar results have been shown by Takeuchi et al.2005; that damaging mice mitochondria with photoirradiating chloromethyl-X-rosamine consistently inhibits GVBD, mitotic spindle formation and the extrusion of polar body. As the increase in the concentration of rotenone, maturation rate decreased gradually, indicating that effect of rotenone to oocyte maturation appeared to be a linear correlation, with no threshold dose.

Embryo fragmentation rate did not differ between groups with different rotenone concentration in both vitrified and unvitrfied groups, however, the fragmentation rates were higher in vitrified groups comparing to fresh groups. The higher fragmentation rate of oocytes undergoing vitrification may be related to the excess of oxidative stress and the toxic effect of cryoprotectants during vitrification (Huang et al., 2008).

Blastocyst rates were comparable when the rotenone concentration was lower than 4 μ M, and significantly decreased at higher rotenone concentration. It seems that there may be a threshold for energy supply to ensure the meiotic spindle function, hence the aneuploidy could be controlled under a certain level for the oocyte's ability to be fertilized and to

develop. The same postulation has been proposed in the case of the decreased fertility in aged women: as the mtDNA mutations accumulate in the oocyte, the frequency of aneuploidy increase once a threshold of mitochondrial energy deficit is crossed (Schon et al., 2000).

Reports have shown that vitrified oocytes showed an increased abnormality in spindle and chromosome configuration, resulted by depolymerization of meiotic spindles and disappearance of microtubules organizing centres (Aman and Parks, 1994, Saunders and Parks, 1999, Webb et al., 1986). Microtubules disassemble partially or completely when oocytes were exposed to a lower than room temperature (Wang et al., 2001, Almeida and Bolton, 1995, Aman and Parks, 1994). For human oocytes, around 50% spindle disassembles after 10 min at room temperature, and 100% after 30min at room temperature accompanied with reduced size of the spindle, and increased chromosome abnormalities (Pickering et al., 1990). After warming, the microtubules tends to repolymerize during the 2-3 hours of incubation (Aigner et al., 1992, Chen et al., 2001), and could be restored in many, but not all of the case (Pickering and Johnson, 1987). Fertilization of oocytes with disrupted spindle could lead to aneuploidy, disgeny, arrested cleavage. (Chen et al., 2003), and lower embryonic development rates, in mice (Huang et al., 2008), and porcine (Wu et al., 2006). In addition, due to the toxicity of the cryoprotectants, high concentration or longer exposure to cryoprotectants could also alter the structure of meiotic spindles (Joly et al., 1992, Van der Elst et al., 1992).

In our study, under the rotenone concentration of 2 μM , even though there were no significant difference in cleavage, fragmentation, and development outcome in fresh oocytes, cryo-survival rate was significantly decrease from 93.3% (control) to 55.6% (2 μM rotenone) after vitrification. The amount of embryos reached 4-8 cell were dropped significantly comparing to control (from 50% to 17.5%). After vitrification, a sufficient restoration of cytoskeleton is essential for chromatid segregation during second meiotic division and the polarity of the mature oocyte (Joly et al., 1992). It is possible that due to the suboptimal of mitochondrial function, the repolymerization of the meiotic spindle and motor proteins function were affected so that the spindle function could not be restored to a sufficient level. When the damage of the microtubule is severe enough, oocyte survivability and subsequent embryonic development after warming is significantly affected.

The model for the disturbance of mitochondria function during IVM in part imitates what happens in oocytes from aged women, which have been shown to have compromised mitochondrial function due to the accumulation of mtDNA mutation and cytoplasmic dysfunction (Shigenaga et al., 1994, Barritt et al., 2000). Our results showed a decrease in cryo-survival rate and early embryonic development in oocytes matured in a rotenone concentration that did not affect maturation. It is concomitant to the lower cryo-survival rate and embryonic development reported in aged oocytes after vitrification. Overall, our study provided an evidence that the compromised mitochondrial function may be one of the main reasons responsible for the lower vitrification outcome of oocytes with

suboptimal cytoplasmic condition, such as oocytes obtained from women with advanced reproductive age.

Chapter 5: Conclusion

5.1 Summary of Contributions

In this thesis, we altered oocyte maturation conditions in vitro to see how they could affect the vitrification outcome of those IVM oocytes. Firstly, the effect of antioxidant supplementation during IVM was studied. We found a significant lower level of fragmentation on the second day of embryonic development in those vitrified MII oocytes matured in the presence of cysteamine. The outcome may be multi-cost, and one of the possible underlying mechanisms we tested was the mtDNA copy number at the end of IVM upon the presence of cysteamine. We proved that the mtDNA replication during IVM was not affected upon the supplementation of cysteamine. Hence, the effect of cysteamine on oocyte maturation in vitro may not be directly related to mtDNA replication and other mechanisms are to be discovered. In the second part of the project, we showed a dose dependent decrease of maturation rate, and embryonic development after fertilization upon the addition of mitochondrial blocker, rotenone. This demonstrated the importance of mitochondrial function during IVM on early embryonic events after fertilization. To our knowledge, we are the first group to use rotenone as a mitochondrial blocker for mouse oocytes and demonstrated a detailed dose dependent impact on oocyte viability. Future more, under the rotenone concentration of 2 μM , even though there were no significant difference in cleavage, fragmentation, and development rate in fresh oocytes, cryo-survival rate was significantly decreased from 93.3% (control) to 55.6% (2 μM rotenone) after vitrification, and the amount of embryos reached 4-8 cell dropped

significantly compared to the control. The model of the disturbance of mitochondrial function during IVM in part imitates what happened in oocytes from aged women, which have been shown to have compromised mitochondrial function. The decrease of cryo-survival rate and early embryonic development in oocytes matured under the presence of rotenone was concomitant to the lower cryopreservation outcome reported from aged oocytes.

Our work indicates that as two individual ART applications, the understanding and improvement of IVM could facilitate the vitrification outcome since the impact of IVM on the oocytes could significantly influence the oocytes' ability to survive and develop following vitrification.

5.2 Future Work

Effect of cysteamine supplementation during IVM on its cryopreservation outcome

We have demonstrated that the addition of cysteamine during IVM could benefit embryonic development of vitrified oocytes by significantly reduce the fragmentation during embryonic development in vitrified oocytes. To investigate the underlying mechanism, mtDNA copy number was tested to see if the change of redox state during IVM could benefit the mtDNA accumulation. While no relationship was found between the cysteamine supplementation and mtDNA accumulation during IVM, other possible mechanisms might be interesting to be looked at. Embryo protection against free oxygen radicals, in part, upon an endogenous pool of antioxidant enzymes stored as mRNA in the

oocyte during oogenesis (Harvey et al., 1995). It has been proposed that the variation in maternal mRNA synthesis or accumulation during oocyte maturation may affect the in-vitro development of embryo until zygotic gene activation (Telford et al., 1990). Hence, a direct approach will be to look at the antioxidant gene expression profile of fresh and post-thawed oocytes matured in the presence or absent of cysteamine. Among the five antioxidant enzyme mRNA transcripts in mouse oocytes suggested by S.El Mouatassim et al, (El Mouatassim et al., 1999), glutathione peroxidase (GPX) and γ -glutamyl-cysteine synthetase(GCS) can be given special interest since their roles in glutathione synthesis and glutathione related oxidative scavenging. We anticipate that if freezing and thawing induce an oxidative stress in the oocytes, a difference in the antioxidant enzyme gene expression between and after vitrification should be seen, and the addition of cysteamine should shift the antioxidant gene expression, especially GPX and GCS, which are involved in the cysteamine function pathway.

Disturbance of mitochondrial function during oocyte maturation on the survival and embryonic development after vitrification:

In the study, we have found a dose dependent decrease in cryo-survival and embryonic development after vitrification of rotenone caused disturbance of mitochondrial function. The hypothesis is that the defect in energy production resulted by mitochondrial malfunction increases oocytes aneuploidy. The same phenomenon has been reported in case of aged related cytoplasmic deficiency. Future studies could be done to assess the change in the cytoplasmic in matured oocytes results from the mitochondria disturbance.

One is to assess the chromosome integrity by chromosome staining of rotenone treated oocytes, before and after vitrification. This could directly shown whether mitochondrial dysfunction result an increase in aneuploidy in the oocytes.

During the process of GVBD and early cleavage of fertilized oocytes, mitochondria are clustered around the nucleus region (Van Blerkom and Runner, 1984). Studies have suggested this pattern of mitochondrial distribution is essential for cellular activities. Visualization of mitochondria distribution of rotenone treated oocytes could reveal if the lower ATP could interrupt this redistribution of the mitochondria.

Appendix I: IVM medium preparation

To have fresh medium for each preparation, we stored concentrated stock solutions in the -20 degree. Concentrated stock solutions were diluted to make 100ml of 1st working solution, which was stored at 4 degree, and can be used for 1 month. 2nd working solution was normally made in a volume of 10ml. It was composed of 1st solution with the addition of Glutamine, Sodium Pyruvate, amino acids and vitamin solution. It was stored in 4 degree, and should be used within 1 week for each preparation. The 3rd working solution was normally prepared on the Day of culture in small volume (1-2 ml). It is prepared by adding hormone, FBS, growth factors into the 2nd working solution. 50 µl IVM medium drops were made in a 35mm petri dish, covered with mineral oil.

IVM protocol

Stock A: (10x)
--100ml

NaCl -	6.800g
KCl -	0.400g
NaH ₂ PO ₄ –	0.109g
CaCl ₂ .2H ₂ O –	0.265g
MgSO ₄ .7H ₂ O -	0.201g
D-Glucose -	1.000g

Stock B: (10X) NaHCO₃ - 1.250g in 100ml

Stock C: (100X) Na-Pyruvate - 0.110 in 10ml

Stock D: (100X)	Glutamine -	0.292 in 10ml
Stock E: (100X)	Penicillin -	0.063g in 10ml
Stock F: (100X)	Streptomycin -	0.05g in 10ml

For 100ml of 1st IVM working solution:

10ml of stock A
 10ml of stock B
 1ml of stock E
 1ml of stock F

Add embryo water to 95ml, phenol red, and gas the mixture. Filter the medium for sterilization.

For 10ml of 2nd working solution

Take 9.5ml of working solution, under the hood, add:

100 µl stock C and D
 200 µl of Essential Amino Acid
 100 µl of Non-essential Amino Acid
 100 µl of Vitamin solution

For the 3rd working solution (on the day of use):

In a volume of 1 ml
 900 µl working solution
 100 µl FBS
 10 µl FSH+LH
 1 µl EGF

Appendix II: Washing medium (mHTF-HEPES)*

	200 ml	1000 ml
CaCl ₂ •2H ₂ O	0.0600 g	0.3000 g
MgSO ₄ •7H ₂ O	0.0099	0.0492 g
NaCl	1.1875 g	5.9375 g
KCl	0.0699 g	0.3496 g
KH ₂ PO ₄	0.0101 g	0.0504 g
NaHCO ₃	0.0920 g	0.4600 g
HEPES	0.9600 g	4.8000 g
Glucose	0.1000 g	0.5000 g
Na-pyruvate	0.0073 g	0.0365 g
L-glutamine	0.0584 g	0.2920 g
Na-lactate	0.6830 ml	3.4200 ml
Penicillin	0.0150 g	0.0750 g
Streptomycin	0.0100 g	0.0500 g
Phenol red (1%)	0.0400 ml	0.2000 ml

* Containing 1mg BSA/ml

Appendix III: Fertilization and Sperm Capacitation medium (mHTF)*

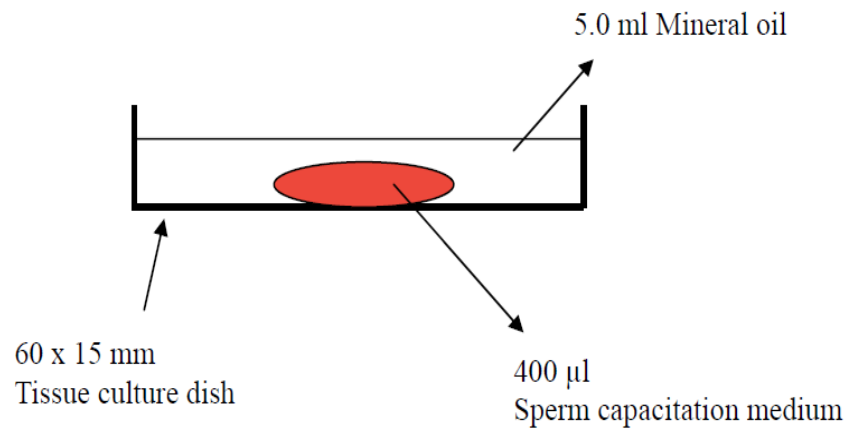
	200ml	1000ml
CaCl ₂ •2H ₂ O	0.0600g	0.3000g
MgSO ₄ •7H ₂ O	0.0099g	0.0492g
NaCl	1.1875g	5.9375g
KCl	0.0699g	0.3496g
KH ₂ PO ₄	0.0101g	0.0504g
NaHCO ₃	0.4200g	2.1000g
Glucose	0.1000g	0.5000g
Na-pyruvate	0.0073g	0.0365g
L-glutamine	0.0584g	0.2920g
Na-lactate	0.6830ml	3.4200ml
Penicillin	0.0150g	0.0750g
Streptomycin	0.0100g	0.0500g
Phenol red (1%)	0.0400ml	0.200ml

* Fertilization medium: 4mg BSA/ml; Sperm capacitation medium: 9mg BSA/ml

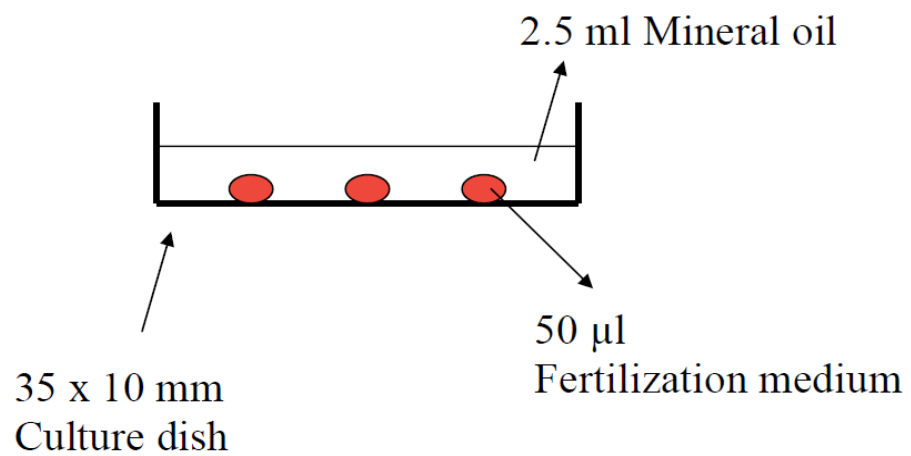
Appendix IV: Preparation of culture dishes

Culture dishes were normally prepared the day before or at least 2 hours prior to use.

Dishes were pre-equilibrated in the incubator.



Capacitation dish was made by 400 μ l of sperm capacitation medium covered with mineral oil



Fertilization, IVM and Development dishes were made of 5 of 50 μ l drops covered with mineral oil

Bibliography

- AHN, H. J., SOHN, I. P., KWON, H. C., JO, D. H., PARK, Y. D. & MIN, C. K. 2002. Characteristics of the cell membrane fluidity, actin fibers, and mitochondrial dysfunctions of frozen-thawed two-cell mouse embryos. *Mol Reprod Dev*, 61, 466-76.
- AIGNER, S., VAN DER ELST, J., SIEBZEHRUBEL, E., WILDT, L., LANG, N. & VAN STEIRTEGHEM, A. C. 1992. The influence of slow and ultra-rapid freezing on the organization of the meiotic spindle of the mouse oocyte. *Hum Reprod*, 7, 857-64.
- AIKEN, C. 2008. Variations in mouse mitochondrial DNA copy number from fertilization to birth are associated with oxidative stress. *Reproductive BioMedicine Online*, 17.
- ALMEIDA, P. A. & BOLTON, V. N. 1995. The effect of temperature fluctuations on the cytoskeletal organisation and chromosomal constitution of the human oocyte. *Zygote*, 3, 357-65.
- AMAN, R. R. & PARKS, J. E. 1994. Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. *Biol Reprod*, 50, 103-10.
- ANAND, T., KUMAR, D., CHAUHAN, M. S., MANIK, R. S. & PALTA, P. 2008. Cysteamine supplementation of in vitro maturation medium, in vitro culture medium or both media promotes in vitro development of buffalo (*Bubalus bubalis*) embryos. *Reprod Fertil Dev*, 20, 253-7.
- BALABAN, R. S., NEMOTO, S. & FINKEL, T. 2005. Mitochondria, oxidants, and aging. *Cell*, 120, 483-95.
- BALASUBRAMANIAN, S. & RHO, G. J. 2007. Effect of cysteamine supplementation of in vitro matured bovine oocytes on chilling sensitivity and development of embryos. *Anim Reprod Sci*, 98, 282-92.
- BANNAI, S. 1984. Transport of cystine and cysteine in mammalian cells. *Biochim Biophys Acta*, 779, 289-306.
- BARHAM, S. S. & BRINKLEY, B. R. 1976. Action of rotenone and related respiratory inhibitors on mammalian cell division. 1 Cell kinetics and biochemical aspects. *Cytobios*, 15, 85-96.
- BARRITT, J. A., COHEN, J. & BRENNER, C. A. 2000. Mitochondrial DNA point mutation in human oocytes is associated with maternal age. *Reprod Biomed Online*, 1, 96-100.
- BAVISTER, B. D. 1995. Culture of preimplantation embryos: facts and artifacts. *Hum Reprod Update*, 1, 91-148.
- BEERMANN, F. & HANSMANN, I. 1986. Follicular maturation, luteinization and first meiotic division in oocytes after inhibiting mitochondrial function in mice with chloramphenicol. *Mutat Res*, 160, 47-54.
- BING, Y. Z., HIRAO, Y., IGA, K., CHE, L. M., TAKENOUCHI, N., KUWAYAMA, M., FUCHIMOTO, D., RODRIGUEZ-MARTINEZ, H. & NAGAI, T. 2002. In vitro maturation and glutathione synthesis of porcine oocytes in the presence or absence of cysteamine under different oxygen tensions: role of cumulus cells. *Reprod Fertil Dev*, 14, 125-31.
- BING, Y. Z., NAGA, T. & RODRIGUEZ-MARTINEZ, H. 2001. Effects of cysteamine, fsh and estradiol-17beta on in vitro maturation of porcine oocytes. *Theriogenology*, 55, 867-76.
- BOGLIOLO, L., LEONI, G., LEDDA, S., NAITANA, S., ZEDDA, M., CARLUCCIO, A. & PAU, S. 2001. Intracytoplasmic sperm injection of in vitro matured oocytes of domestic cats with frozen-thawed epididymal spermatozoa. *Theriogenology*, 56, 955-67.
- BUGGE, M., COLLINS, A., PETERSEN, M. B., FISHER, J., BRANDT, C., HERTZ, J. M., TRANEBJAERG, L., DE LOZIER-BLANCHET, C., NICOLAIDES, P., BRONDUM-NIELSEN, K., MORTON, N. & MIKKELSEN, M.

1998. Non-disjunction of chromosome 18. *Hum Mol Genet*, 7, 661-9.
- CAO, L., SHITARA, H., HORII, T., NAGAO, Y., IMAI, H., ABE, K., HARA, T., HAYASHI, J. & YONEKAWA, H. 2007. The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells. *Nat Genet*, 39, 386-90.
- CAO, Y. X. & CHIAN, R. C. 2009. Fertility preservation with immature and in vitro matured oocytes. *Semin Reprod Med*, 27, 456-64.
- CHA, K. Y., KOO, J. J., KO, J. J., CHOI, D. H., HAN, S. Y. & YOON, T. K. 1991. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertil Steril*, 55, 109-13.
- CHANG, C. C., SHAPIRO, D. B., BERNAL, D. P., WRIGHT, G., KORT, H. I. & NAGY, Z. P. 2008. Human oocyte vitrification: in-vivo and in-vitro maturation outcomes. *Reprod Biomed Online*, 17, 684-8.
- CHEN, N., LIOW, S. L., YIP, W. Y., TAN, L. G. & NG, S. C. 2005. Influence of cysteamine supplementation and culture in portable dry-incubator on the in vitro maturation, fertilization and subsequent development of mouse oocytes. *Theriogenology*, 63, 2300-10.
- CHEN, S. U., LIEN, Y. R., CHAO, K. H., HO, H. N., YANG, Y. S. & LEE, T. Y. 2003. Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing--a review article. *Mol Cell Endocrinol*, 202, 101-7.
- CHEN, S. U., LIEN, Y. R., CHEN, H. F., CHAO, K. H., HO, H. N. & YANG, Y. S. 2000. Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws. *Hum Reprod*, 15, 2598-603.
- CHEN, S. U., LIEN, Y. R., CHENG, Y. Y., CHEN, H. F., HO, H. N. & YANG, Y. S. 2001. Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straws (OPS) and grids. *Hum Reprod*, 16, 2350-6.
- CHIAN, R. C., BUCKETT, W. M., ABDUL JALIL, A. K., SON, W. Y., SYLVESTRE, C., RAO, D. & TAN, S. L. 2004. Natural-cycle in vitro fertilization combined with in vitro maturation of immature oocytes is a potential approach in infertility treatment. *Fertil Steril*, 82, 1675-8.
- CHIAN, R. C., BUCKETT, W. M., TOO, L. L. & TAN, S. L. 1999. Pregnancies resulting from in vitro matured oocytes retrieved from patients with polycystic ovary syndrome after priming with human chorionic gonadotropin. *Fertil Steril*, 72, 639-42.
- CHIAN, R. C., HUANG, J. Y., GILBERT, L., SON, W. Y., HOLZER, H., CUI, S. J., BUCKETT, W. M., TULANDI, T. & TAN, S. L. 2009. Obstetric outcomes following vitrification of in vitro and in vivo matured oocytes. *Fertil Steril*, 91, 2391-8.
- CHIAN, R. C., HUANG, J. Y., TAN, S. L., LUCENA, E., SAA, A., ROJAS, A., RUVALCABA CASTELLON, L. A., GARCIA AMADOR, M. I. & MONTROYA SARMIENTO, J. E. 2008. Obstetric and perinatal outcome in 200 infants conceived from vitrified oocytes. *Reprod Biomed Online*, 16, 608-10.
- COMBELLES, C. M., GUPTA, S. & AGARWAL, A. 2009. Could oxidative stress influence the in-vitro maturation of oocytes? *Reprod Biomed Online*, 18, 864-80.
- COOPER, A., PAYNTER, S. J., FULLER, B. J. & SHAW, R. W. 1998. Differential effects of cryopreservation on nuclear or cytoplasmic maturation in vitro in immature mouse oocytes from stimulated ovaries. *Hum Reprod*, 13, 971-8.
- DE MATOS, D. G. & FURNUS, C. C. 2000. The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. *Theriogenology*, 53, 761-71.

- DE MATOS, D. G., FURNUS, C. C. & MOSES, D. F. 1997. Glutathione synthesis during in vitro maturation of bovine oocytes: role of cumulus cells. *Biol Reprod*, 57, 1420-5.
- DE MATOS, D. G., FURNUS, C. C., MOSES, D. F. & BALDASSARRE, H. 1995. Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured in vitro. *Mol Reprod Dev*, 42, 432-6.
- DE MATOS, D. G., FURNUS, C. C., MOSES, D. F., MARTINEZ, A. G. & MATKOVIC, M. 1996. Stimulation of glutathione synthesis of in vitro matured bovine oocytes and its effect on embryo development and freezability. *Mol Reprod Dev*, 45, 451-7.
- DE MATOS, D. G., GASPARRINI, B., PASQUALINI, S. R. & THOMPSON, J. G. 2002a. Effect of glutathione synthesis stimulation during in vitro maturation of ovine oocytes on embryo development and intracellular peroxide content. *Theriogenology*, 57, 1443-51.
- DE MATOS, D. G., HERRERA, C., CORTVRINDT, R., SMITZ, J., VAN SOOM, A., NOGUEIRA, D. & PASQUALINI, R. S. 2002b. Cysteamine supplementation during in vitro maturation and embryo culture: a useful tool for increasing the efficiency of bovine in vitro embryo production. *Mol Reprod Dev*, 62, 203-9.
- DE MATOS, D. G., NOGUEIRA, D., CORTVRINDT, R., HERRERA, C., ADRIAENSSENS, T., PASQUALINI, R. S. & SMITZ, J. 2003. Capacity of adult and prepubertal mouse oocytes to undergo embryo development in the presence of cysteamine. *Mol Reprod Dev*, 64, 214-8.
- DELEUZE, S., DUBOIS, C. S., CAILLAUD, M., BRUNEAU, B., GOUDET, G. & DUCHAMP, G. 2010. Influence of cysteamine on in vitro maturation, in vitro and in vivo fertilization of equine oocytes. *Reprod Domest Anim*, 45, 1-7.
- DELEUZE, S. & GOUDET, G. 2010. Cysteamine Supplementation of In vitro Maturation Media: A Review. *Reprod Domest Anim*.
- DELL'AQUILA, M. E., AMBRUOSI, B., DE SANTIS, T. & CHO, Y. S. 2009. Mitochondrial distribution and activity in human mature oocytes: gonadotropin-releasing hormone agonist versus antagonist for pituitary down-regulation. *Fertil Steril*, 91, 249-55.
- DENEKE, S. M. & FANBURG, B. L. 1989. Regulation of cellular glutathione. *Am J Physiol*, 257, L163-73.
- DINARA, S., SENGOKU, K., TAMATE, K., HORIKAWA, M. & ISHIKAWA, M. 2001. Effects of supplementation with free radical scavengers on the survival and fertilization rates of mouse cryopreserved oocytes. *Hum Reprod*, 16, 1976-81.
- DUMOLLARD, R., CARROLL, J., DUCHEN, M. R., CAMPBELL, K. & SWANN, K. 2009. Mitochondrial function and redox state in mammalian embryos. *Semin Cell Dev Biol*, 20, 346-53.
- DUMOLLARD, R., WARD, Z., CARROLL, J. & DUCHEN, M. R. 2007. Regulation of redox metabolism in the mouse oocyte and embryo. *Development*, 134, 455-65.
- EBERT, K. M., LIEM, H. & HECHT, N. B. 1988. Mitochondrial DNA in the mouse preimplantation embryo. *J Reprod Fertil*, 82, 145-9.
- EICHENLAUB-RITTER, U., BAART, E., YIN, H. & BETZENDAHL, I. 1996. Mechanisms of spontaneous and chemically-induced aneuploidy in mammalian oogenesis: basis of sex-specific differences in response to aneugens and the necessity for further tests. *Mutat Res*, 372, 279-94.
- EL MOUATASSIM, S., GUERIN, P. & MENEZO, Y. 1999. Expression of genes encoding antioxidant enzymes in human and mouse oocytes during the final stages of maturation. *Mol Hum Reprod*, 5, 720-5.
- FEUGANG, J. M., DE ROOVER, R., MOENS, A., LEONARD, S., DESSY, F. & DONNAY, I. 2004. Addition of beta-mercaptoethanol or Trolox at the morula/blastocyst stage improves the quality of bovine blastocysts and prevents induction of apoptosis and degeneration by prooxidant agents. *Theriogenology*, 61, 71-90.

- FUNAHASHI, H., BANDO, N., NAKAHIRA, S., OH, S. H. & TSUBOI, S. 1999. Changes in intracellular content of glutathione and thiols associated with gamma-glutamyl cycle during sperm penetration and pronuclear formation in rat oocytes. *Zygote*, 7, 301-5.
- GABRIELSEN A, F. J., AGERHOLM I 2004. Parameters predicting the implantation rate of thawed IVF/ICSI embryos: a retrospective study. *reprod Biomed Online*, 12, 70-6.
- GARDINER, C. S. & REED, D. J. 1994. Status of glutathione during oxidant-induced oxidative stress in the preimplantation mouse embryo. *Biol Reprod*, 51, 1307-14.
- GASPARRINI, B., NEGLIA, G., PALO, R. D., CAMPANILE, G. & ZICARELLI, L. 2000. Effect of cysteamine during in vitro maturation on buffalo embryo development. *Theriogenology*, 54, 1537-42.
- GASPARRINI, B., SAYOUD, H., NEGLIA, G., MATOS, D. G., DONNAY, I. & ZICARELLI, L. 2003. Glutathione synthesis during in vitro maturation of buffalo (*Bubalus bubalis*) oocytes: effects of cysteamine on embryo development. *Theriogenology*, 60, 943-52.
- GIBSON, T. C., KUBISCH, H. M. & BRENNER, C. A. 2005. Mitochondrial DNA deletions in rhesus macaque oocytes and embryos. *Mol Hum Reprod*, 11, 785-9.
- GOTT, A. L., HARDY, K., WINSTON, R. M. & LEESE, H. J. 1990. Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Hum Reprod*, 5, 104-8.
- GRUPEN, C. G., NAGASHIMA, H. & NOTTLE, M. B. 1995. Cysteamine enhances in vitro development of porcine oocytes matured and fertilized in vitro. *Biol Reprod*, 53, 173-8.
- GUERIN, P., EL MOUATASSIM, S. & MENEZO, Y. 2001. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update*, 7, 175-89.
- HARVEY, M. B., ARCELLANA-PANLILIO, M. Y., ZHANG, X., SCHULTZ, G. A. & WATSON, A. J. 1995. Expression of genes encoding antioxidant enzymes in preimplantation mouse and cow embryos and primary bovine oviduct cultures employed for embryo coculture. *Biol Reprod*, 53, 532-40.
- HASSOLD, T. & CHIU, D. 1985. Maternal age-specific rates of numerical chromosome abnormalities with special reference to trisomy. *Hum Genet*, 70, 11-7.
- HASSOLD, T. & HUNT, P. 2001. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet*, 2, 280-91.
- HOLZER, H., SCHARF, E., CHIAN, R. C., DEMIRTAS, E., BUCKETT, W. & TAN, S. L. 2007. In vitro maturation of oocytes collected from unstimulated ovaries for oocyte donation. *Fertil Steril*, 88, 62-7.
- HONG, S. W., CHUNG, H. M., LIM, J. M., KO, J. J., YOON, T. K., YEE, B. & CHA, K. Y. 1999. Improved human oocyte development after vitrification: a comparison of thawing methods. *Fertil Steril*, 72, 142-6.
- HOSSEIN, M. S., KIM, M. K., JANG, G., OH, H. J., KOO, O., KIM, J. J., KANG, S. K., LEE, B. C. & HWANG, W. S. 2007. Effects of thiol compounds on in vitro maturation of canine oocytes collected from different reproductive stages. *Mol Reprod Dev*, 74, 1213-20.
- HOSSEINI, S. M., FOROUZANFAR, M., HAJIAN, M., ASGARI, V., ABEDI, P., HOSSEINI, L., OSTADHOSSEINI, S., MOULAVI, F., SAFAHANI LANGRROODI, M., SADEGHI, H., BAHRAMIAN, H., EGHBALSAIED, S. & NASR-ESFAHANI, M. H. 2009. Antioxidant supplementation of culture medium during embryo development and/or after vitrification-warming; which is the most important? *J Assist Reprod Genet*, 26, 355-64.
- HUANG, J. Y., CHEN, H. Y., PARK, J. Y., TAN, S. L. & CHIAN, R. C. 2008. Comparison of spindle and chromosome configuration in in vitro- and in vivo-matured mouse oocytes after vitrification. *Fertil Steril*, 90, 1424-32.

- ISSELS, R. D., NAGELE, A., ECKERT, K. G. & WILMANN, W. 1988. Promotion of cystine uptake and its utilization for glutathione biosynthesis induced by cysteamine and N-acetylcysteine. *Biochem Pharmacol*, 37, 881-8.
- JOLY, C., BICHINI, O., BOULEKBACHE, H., TESTART, J. & MARO, B. 1992. Effects of 1,2-propanediol on the cytoskeletal organization of the mouse oocyte. *Hum Reprod*, 7, 374-8.
- KOBAYASHI, M., ASAKUMA, S. & FUKUI, Y. 2007. Blastocyst production by in vitro maturation and development of porcine oocytes in defined media following intracytoplasmic sperm injection. *Zygote*, 15, 93-102.
- KOBAYASHI, M., LEE, E. S. & FUKUI, Y. 2006. Cysteamine or beta-mercaptoethanol added to a defined maturation medium improves blastocyst formation of porcine oocytes after intracytoplasmic sperm injection. *Theriogenology*, 65, 1191-9.
- KULESHOVA, L., GIANAROLI, L., MAGLI, C., FERRARETTI, A. & TROUNSON, A. 1999. Birth following vitrification of a small number of human oocytes: case report. *Hum Reprod*, 14, 3077-9.
- LAVERGE, H., VAN DER ELST, J., DE SUTTER, P., VERSCHRAEGEN-SPAE, M. R., DE PAEPE, A. & DHONT, M. 1998. Fluorescent in-situ hybridization on human embryos showing cleavage arrest after freezing and thawing. *Hum Reprod*, 13, 425-9.
- LONERGAN, P., GUTIERREZ-ADAN, A., RIZOS, D., PINTADO, B., DE LA FUENTE, J. & BOLAND, M. P. 2003. Relative messenger RNA abundance in bovine oocytes collected in vitro or in vivo before and 20 hr after the preovulatory luteinizing hormone surge. *Mol Reprod Dev*, 66, 297-305.
- LUBERDA, Z. 2005. The role of glutathione in mammalian gametes. *Reprod Biol*, 5, 5-17.
- LUCENA, E., BERNAL, D. P., LUCENA, C., ROJAS, A., MORAN, A. & LUCENA, A. 2006. Successful ongoing pregnancies after vitrification of oocytes. *Fertil Steril*, 85, 108-11.
- LUCIANO, A. M., GOUDET, G., PERAZZOLI, F., LAHUEC, C. & GERARD, N. 2006. Glutathione content and glutathione peroxidase expression in in vivo and in vitro matured equine oocytes. *Mol Reprod Dev*, 73, 658-66.
- LUCIANO, A. M., LODDE, V., BERETTA, M. S., COLLEONI, S., LAURIA, A. & MODINA, S. 2005. Developmental capability of denuded bovine oocyte in a co-culture system with intact cumulus-oocyte complexes: role of cumulus cells, cyclic adenosine 3',5'-monophosphate, and glutathione. *Mol Reprod Dev*, 71, 389-97.
- MAEDOMARI, N., KIKUCHI, K., OZAWA, M., NOGUCHI, J., KANEKO, H., OHNUMA, K., NAKAI, M., SHINO, M., NAGAI, T. & KASHIWAZAKI, N. 2007. Cytoplasmic glutathione regulated by cumulus cells during porcine oocyte maturation affects fertilization and embryonic development in vitro. *Theriogenology*, 67, 983-93.
- MEIER, T. & ISSELS, R. D. 1995. Promotion of cyst(e)ine uptake. *Methods Enzymol*, 252, 103-12.
- MEISTER, A. 1983. Selective modification of glutathione metabolism. *Science*, 220, 472-7.
- NASR-ESFAHANI, M. H. & JOHNSON, M. H. 1992. Quantitative analysis of cellular glutathione in early preimplantation mouse embryos developing in vivo and in vitro. *Hum Reprod*, 7, 1281-90.
- NISHI, Y., TAKESHITA, T., SATO, K. & ARAKI, T. 2003. Change of the mitochondrial distribution in mouse ooplasm during in vitro maturation. *J Nippon Med Sch*, 70, 408-15.
- ODANI, M., KOMATSU, Y., OKA, S. & IWAHASHI, H. 2003. Screening of genes that respond to cryopreservation stress using yeast DNA microarray. *Cryobiology*, 47, 155-64.
- OKIMURA T, K. K., ZHAN Q, ET AL 2005. Update on clinical efficiency of the vitrification method for human oocytes in an in vitro fertilization program. *Fertil Steril*, 84(suppl 1), S174.
- ORRENIUS, S., GOGVADZE, V. & ZHIVOTOVSKY, B. 2007. Mitochondrial oxidative stress: implications for cell

- death. *Annu Rev Pharmacol Toxicol*, 47, 143-83.
- OYAMADA, T. & FUKUI, Y. 2004. Oxygen tension and medium supplements for in vitro maturation of bovine oocytes cultured individually in a chemically defined medium. *J Reprod Dev*, 50, 107-17.
- PAN, H., MA, P., ZHU, W. & SCHULTZ, R. M. 2008. Age-associated increase in aneuploidy and changes in gene expression in mouse eggs. *Dev Biol*, 316, 397-407.
- PELLESTOR, F., ANDREO, B., ARNAL, F., HUMEAU, C. & DEMAILLE, J. 2002. Mechanisms of non-disjunction in human female meiosis: the co-existence of two modes of malsegregation evidenced by the karyotyping of 1397 in-vitro unfertilized oocytes. *Hum Reprod*, 17, 2134-45.
- PICKERING, S. J., BRAUDE, P. R., JOHNSON, M. H., CANT, A. & CURRIE, J. 1990. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil Steril*, 54, 102-8.
- PICKERING, S. J. & JOHNSON, M. H. 1987. The influence of cooling on the organization of the meiotic spindle of the mouse oocyte. *Hum Reprod*, 2, 207-16.
- PIKO, L. & TAYLOR, K. D. 1987. Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Dev Biol*, 123, 364-74.
- PLACHOT, M. 2003. Genetic analysis of the oocyte--a review. *Placenta*, 24 Suppl B, S66-9.
- REYNIER, P., MAY-PANLOUP, P., CHRETIEN, M. F., MORGAN, C. J., JEAN, M., SAVAGNER, F., BARRIERE, P. & MALTHIERY, Y. 2001. Mitochondrial DNA content affects the fertilizability of human oocytes. *Mol Hum Reprod*, 7, 425-9.
- RICHTER, C., SUTER, M. & WALTER, P. B. 1998. Mitochondrial free radical damage and DNA repair. *Biofactors*, 7, 207-8.
- RODRIGUEZ-GONZALEZ, E., LOPEZ-BEJAR, M., IZQUIERDO, D. & PARAMIO, M. T. 2003a. Developmental competence of prepubertal goat oocytes selected with brilliant cresyl blue and matured with cysteamine supplementation. *Reprod Nutr Dev*, 43, 179-87.
- RODRIGUEZ-GONZALEZ, E., LOPEZ-BEJAR, M., MERTENS, M. J. & PARAMIO, M. T. 2003b. Effects on in vitro embryo development and intracellular glutathione content of the presence of thiol compounds during maturation of prepubertal goat oocytes. *Mol Reprod Dev*, 65, 446-53.
- SANTOS, T. A., EL SHOURBAGY, S. & ST JOHN, J. C. 2006. Mitochondrial content reflects oocyte variability and fertilization outcome. *Fertil Steril*, 85, 584-91.
- SATOH, M. & KUROIWA, T. 1991. Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. *Exp Cell Res*, 196, 137-40.
- SAUNDERS, K. M. & PARKS, J. E. 1999. Effects of cryopreservation procedures on the cytology and fertilization rate of in vitro-matured bovine oocytes. *Biol Reprod*, 61, 178-87.
- SCHON, E. A., KIM, S. H., FERREIRA, J. C., MAGALHAES, P., GRACE, M., Warburton, D. & GROSS, S. J. 2000. Chromosomal non-disjunction in human oocytes: is there a mitochondrial connection? *Hum Reprod*, 15 Suppl 2, 160-72.
- SHIGENAGA, M. K., HAGEN, T. M. & AMES, B. N. 1994. Oxidative damage and mitochondrial decay in aging. *Proc Natl Acad Sci U S A*, 91, 10771-8.
- SINGHAL, S., PRASAD, S., SINGH, B., PRASAD, J. K. & GUPTA, H. P. 2009. Effect of including growth factors and antioxidants in maturation medium used for in vitro culture of buffalo oocytes recovered in vivo. *Anim Reprod Sci*, 113, 44-50.
- SLOTTE, H., GUSTAFSON, O., NYLUND, L. & POUSETTE, A. 1990. ATP and ADP in human pre-embryos. *Hum Reprod*, 5, 319-22.
- SOMFAI, T., OZAWA, M., NOGUCHI, J., KANEKO, H., KURIANI KARJA, N. W., FARHUDIN, M., DINNYES, A.,

- NAGAI, T. & KIKUCHI, K. 2007. Developmental competence of in vitro-fertilized porcine oocytes after in vitro maturation and solid surface vitrification: effect of cryopreservation on oocyte antioxidative system and cell cycle stage. *Cryobiology*, 55, 115-26.
- SONG, K. & LEE, E. 2007. Modification of maturation condition improves oocyte maturation and in vitro development of somatic cell nuclear transfer pig embryos. *J Vet Sci*, 8, 81-7.
- SPIKINGS, E. C., ALDERSON, J. & ST JOHN, J. C. 2007. Regulated mitochondrial DNA replication during oocyte maturation is essential for successful porcine embryonic development. *Biol Reprod*, 76, 327-35.
- STEUERWALD, N., BARRITT, J. A., ADLER, R., MALTER, H., SCHIMMEL, T., COHEN, J. & BRENNER, C. A. 2000. Quantification of mtDNA in single oocytes, polar bodies and subcellular components by real-time rapid cycle fluorescence monitored PCR. *Zygote*, 8, 209-15.
- STOJKOVIC, M., MACHADO, S. A., STOJKOVIC, P., ZAKHARTCHENKO, V., HUTZLER, P., GONCALVES, P. B. & WOLF, E. 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biol Reprod*, 64, 904-9.
- SUCCU, S., BEBBERE, D., BOGLIOLO, L., ARIU, F., FOIS, S., LEONI, G. G., BERLINGUER, F., NAITANA, S. & LEDDA, S. 2008. Vitrification of in vitro matured ovine oocytes affects in vitro pre-implantation development and mRNA abundance. *Mol Reprod Dev*, 75, 538-46.
- SUN, Q. Y., WU, G. M., LAI, L., PARK, K. W., CABOT, R., CHEONG, H. T., DAY, B. N., PRATHER, R. S. & SCHATTEN, H. 2001. Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro. *Reproduction*, 122, 155-63.
- SUTOVSKY, P. & SCHATTEN, G. 1997. Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. *Biol Reprod*, 56, 1503-12.
- TAKAHASHI, M., NAGAI, T., HAMANO, S., KUWAYAMA, M., OKAMURA, N. & OKANO, A. 1993. Effect of thiol compounds on in vitro development and intracellular glutathione content of bovine embryos. *Biol Reprod*, 49, 228-32.
- TAKEUCHI, T., GONG, J., VEECK, L. L., ROSENWAKS, Z. & PALERMO, G. D. 2001. Preliminary findings in germinal vesicle transplantation of immature human oocytes. *Hum Reprod*, 16, 730-6.
- TAKEUCHI, T., NERI, Q. V., KATAGIRI, Y., ROSENWAKS, Z. & PALERMO, G. D. 2005. Effect of treating induced mitochondrial damage on embryonic development and epigenesis. *Biol Reprod*, 72, 584-92.
- TAN, X., SONG, E., LIU, X., YOU, W. & WAN, F. 2009. Factors affecting the survival, fertilization, and embryonic development of mouse oocytes after vitrification using glass capillaries. *In Vitro Cell Dev Biol Anim*, 45, 420-9.
- TATEMOTO, H., MUTO, N., SUNAGAWA, I., SHINJO, A. & NAKADA, T. 2004. Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid. *Biol Reprod*, 71, 1150-7.
- TATEMOTO, H., SAKURAI, N. & MUTO, N. 2000. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during In vitro maturation: role of cumulus cells. *Biol Reprod*, 63, 805-10.
- TATONE, C., DI EMIDIO, G., VENTO, M., CIRIMINNA, R. & ARTINI, P. G. 2010. Cryopreservation and oxidative stress in reproductive cells. *Gynecol Endocrinol*.
- TELFORD, N. A., WATSON, A. J. & SCHULTZ, G. A. 1990. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol Reprod Dev*, 26, 90-100.

- THOMSON, L. K., FLEMING, S. D., AITKEN, R. J., DE IULIIS, G. N., ZIESCHANG, J. A. & CLARK, A. M. 2009. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod*, 24, 2061-70.
- TOTH, T. L., BAKA, S. G., VEECK, L. L., JONES, H. W., JR., MUASHER, S. & LANZENDORF, S. E. 1994a. Fertilization and in vitro development of cryopreserved human prophase I oocytes. *Fertil Steril*, 61, 891-4.
- TOTH, T. L., LANZENDORF, S. E., SANDOW, B. A., VEECK, L. L., HASSEN, W. A., HANSEN, K. & HODGEN, G. D. 1994b. Cryopreservation of human prophase I oocytes collected from unstimulated follicles. *Fertil Steril*, 61, 1077-82.
- URDANETA, A., JIMENEZ-MACEDO, A. R., IZQUIERDO, D. & PARAMIO, M. T. 2003. Supplementation with cysteamine during maturation and embryo culture on embryo development of prepubertal goat oocytes selected by the brilliant cresyl blue test. *Zygote*, 11, 347-54.
- URDANETA, A., JIMENEZ, A. R., PARAMIO, M. T. & IZQUIERDO, D. 2004. Cysteamine, glutathione and ionomycin treatments improve in vitro fertilization of prepubertal goat oocytes. *Zygote*, 12, 277-84.
- VAN BLERKOM, J. 1991. Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes. *Proc Natl Acad Sci U S A*, 88, 5031-5.
- VAN BLERKOM, J. 2004. Mitochondria in human oogenesis and preimplantation embryogenesis: engines of metabolism, ionic regulation and developmental competence. *Reproduction*, 128, 269-80.
- VAN BLERKOM, J., DAVIS, P. W. & LEE, J. 1995. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. *Hum Reprod*, 10, 415-24.
- VAN BLERKOM, J. & RUNNER, M. N. 1984. Mitochondrial reorganization during resumption of arrested meiosis in the mouse oocyte. *Am J Anat*, 171, 335-55.
- VAN BLERKOM, J., SINCLAIR, J. & DAVIS, P. 1998. Mitochondrial transfer between oocytes: potential applications of mitochondrial donation and the issue of heteroplasmy. *Hum Reprod*, 13, 2857-68.
- VAN DER ELST, J., VAN DEN ABBEEL, E., NERINCKX, S. & VAN STEIRTEGHEM, A. 1992. Parthenogenetic activation pattern and microtubular organization of the mouse oocyte after exposure to 1,2-propanediol. *Cryobiology*, 29, 549-62.
- WANG, W. H., MENG, L., HACKETT, R. J., ODENBOURG, R. & KEEFE, D. L. 2001. Limited recovery of meiotic spindles in living human oocytes after cooling-rewarming observed using polarized light microscopy. *Hum Reprod*, 16, 2374-8.
- WEBB, M., HOWLETT, S. K. & MARO, B. 1986. Parthenogenesis and cytoskeletal organization in ageing mouse eggs. *J Embryol Exp Morphol*, 95, 131-45.
- WHITELEY, G. S., FULLER, B. J. & HOBBS, K. E. 1992. Deterioration of cold-stored tissue specimens due to lipid peroxidation: modulation by antioxidants at high subzero temperatures. *Cryobiology*, 29, 668-73.
- WILDING, M., DALE, B., MARINO, M., DI MATTEO, L., ALVIGGI, C., PISATURO, M. L., LOMBARDI, L. & DE PLACIDO, G. 2001. Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Hum Reprod*, 16, 909-17.
- WU, C., RUI, R., DAI, J., ZHANG, C., JU, S., XIE, B., LU, X. & ZHENG, X. 2006. Effects of cryopreservation on the developmental competence, ultrastructure and cytoskeletal structure of porcine oocytes. *Mol Reprod Dev*, 73, 1454-62.
- YAMAUCHI, N. & NAGAI, T. 1999. Male pronuclear formation in denuded porcine oocytes after in vitro maturation in the presence of cysteamine. *Biol Reprod*, 61, 828-33.

- YOON, P. W., FREEMAN, S. B., SHERMAN, S. L., TAFT, L. F., GU, Y., PETTAY, D., FLANDERS, W. D., KHOURY, M. J. & HASSOLD, T. J. 1996. Advanced maternal age and the risk of Down syndrome characterized by the meiotic stage of chromosomal error: a population-based study. *Am J Hum Genet*, 58, 628-33.
- YOSHIDA, M., ISHIGAKI, K., NAGAI, T., CHIKYU, M. & PURSEL, V. G. 1993. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. *Biol Reprod*, 49, 89-94.
- ZENG, H. T., REN, Z., YEUNG, W. S., SHU, Y. M., XU, Y. W., ZHUANG, G. L. & LIANG, X. Y. 2007. Low mitochondrial DNA and ATP contents contribute to the absence of birefringent spindle imaged with PolScope in in vitro matured human oocytes. *Hum Reprod*, 22, 1681-6.
- ZHANG, J., WANG, C. W., KREY, L., LIU, H., MENG, L., BLASZCZYK, A., ADLER, A. & GRIFO, J. 1999. In vitro maturation of human preovulatory oocytes reconstructed by germinal vesicle transfer. *Fertil Steril*, 71, 726-31.
- ZHOU, P., WU, Y. G., LI, Q., LAN, G. C., WANG, G., GAO, D. & TAN, J. H. 2008. The interactions between cysteamine, cystine and cumulus cells increase the intracellular glutathione level and developmental capacity of goat cumulus-denuded oocytes. *Reproduction*, 135, 605-11.
- ZUELKE, K. A., JEFFAY, S. C., ZUCKER, R. M. & PERREAULT, S. D. 2003. Glutathione (GSH) concentrations vary with the cell cycle in maturing hamster oocytes, zygotes, and pre-implantation stage embryos. *Mol Reprod Dev*, 64, 106-12.