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Effect of methanol and Me_2SO exposure on mitochondrial activity and distribution in stage III ovarian follicles of zebrafish (*Danio rerio*) ☆

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ABSTRACT

In this study the effect of cryoprotectants that have been shown to be the least toxic to zebrafish ovarian follicles (methanol and Me_2SO), on mitochondria of stage III ovarian follicles was evaluated. The mitochondrial distributional arrangement, mitochondrial membrane potential, mtDNA copy number, ATP levels and ADP/ATP ratios were assessed following exposure to cryoprotectants for 30 min at room temperature. Results obtained by confocal microscopy showed that 30 min exposure to 2 M methanol induced a loss of membrane potential, although viability tests showed no decrease in survival even after 5 h post-exposure incubation. Higher concentrations of methanol (3 and 4 M) induced not only a decrease in mitochondrial membrane potential but also the loss of mitochondrial distributional arrangement, which suggested a compromised mitochondrial function. Furthermore 3 and 4 M treatments resulted in a decrease in viability assessed by Fluorescein diacetate–Propidium iodide (FDA–PI) and in a decrease in mtDNA copy number and ADP/ATP ratio after 5 h incubation following methanol exposure, indicating a delayed effect. The use of Me_2SO, which is considered to be a more toxic CPA to zebrafish ovarian follicles than methanol, caused a decrease in viability and a sustained decrease in ATP levels accompanied by failure to maintain mtDNA copy number within 1 h post-exposure incubation. These results indicated that even CPAs that are considered to have no toxicity as determined by Trypan blue (TB) and FDA–PI tests can have a deleterious effect on mitochondrial activity, potentially compromising oocyte growth and embryo development.

Introduction

The zebrafish is an extensively used model in bio-medical research, and one where the lack of successful protocols for cryopreservation of eggs, oocytes and ovarian tissue compromises the use of the species in high throughput germplasm repositories [21]. Mazur and Leibo [21] also point out the importance of investigation of cryobiological fundamentals at a molecular level. An understanding of the sub-cellular events linked to cryoprotocols are clearly needed to inform the development of successful cryopreservation protocols for zebrafish oocytes.

The late ovarian follicle of the zebrafish is a complex system of acellular and cellular structures surrounding the developing egg, the oocyte. The large size of fish ovarian follicles, their high yolk content and their chilling sensitivity [14] have been a barrier to their successful cryopreservation. Previous cryopreservation studies have focused on the use of cryoprotectants (CPAs) to reduce the risk of chilling injury and intracellular ice formation. CPAs can suppress most cryoinjuries but when used at high concentrations, they become toxic to biological material [19].

Detailed information on the toxicities of cryoprotectants is essential for development of cryopreservation protocols for ovarian follicles. To date, the effect of cryoprotectants in zebrafish ovarian follicles has been evaluated by viability tests including staining protocols, Trypan blue (TB), Thyazolyl blue (MTT), Fluorescein diacetate (FDA) and Propidium iodide (PI), and by the observation of germinal vesicle breakdown (GVBD) [25,32]. These tests have known limitations, they are stage-specific as in the case of GVBD or evaluate only one (TB, MTT) or two (FDA–PI) parameters. However, there are other possible subtle impacts of CPA use which may not result in cell death, but can compromise future development.

The detrimental effect of cryoprotectants may be osmotic or due to direct biochemical injury. Osmotic injury results from changes in cell volume which may be induced by cryoprotectant addition or removal [26]. The biochemical injury could be due to direct interaction of cryoprotectant on biomolecules in the cell, e.g. enzymes, or by indirect action of cryoprotectant by altering the environment of cellular biomolecules modifying redox potential, dielectric constant, ionic strength, pH and surface tension [2,3,9]. Mitochondria are particularly susceptible to cryopreservation damage [24], and can mediate both apoptosis and necrosis [10].

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Mitochondria have a wide range of cellular functions that affect metabolism and reproduction. They are responsible for the production of most cellular energy, in the form of ATP, through oxidative phosphorylation, and are also implicated in triggering cell aging and death (apoptosis) following cellular insult. Quantitative variation in mitochondrial DNA (mtDNA) has been associated with gamete quality and reproductive success in other animals [13,28] including humans [5,22]. The number of mitochondria and mitochondrial genomes varies between tissues [23]. Different factors are involved in the maintenance of mtDNA copy number in animal cells, including mitochondrial transcription factor A (TFAM) [7].

The quantity of mitochondria in the oocyte affects its ability to produce ATP [29]. Measurement of ATP content is a commonly used method to quantify cellular energetics as maintenance of high intracellular ATP means that mitochondria and the ATP-generating mechanism are functionally intact.

This study investigates the intact ovarian follicle. Three approaches, ATP levels, ADP/ATP ratios and mtDNA copy number, provide data on the total intact follicle whilst a fourth, the confocal studies with the mitochondrial probe JC-1, only provides information on the granulosa layer of the follicle. We have previously reported the inability of the mitochondrial probe to penetrate the oocyte [33].

The aim of this study was to investigate the effect of methanol, known to be the least toxic cryoprotectant to zebrafish ovarian follicles [14,25], on the mitochondrial distribution and activity in granulosa cells of stage III zebrafish ovarian follicles using confocal microscopy with the mitochondrial probe JC-1. We previously reported that the distributional arrangement of mitochondria in the granulosa cells surrounding stage III zebrafish oocyte [33], obtained by confocal microscopy, showed a contiguous aggregation of mitochondria at the margin of each granulosa cell, with each fluorescence pattern having a diameter of 10–15 μm. Results obtained in the present study showed damaged mitochondrial structure and change in fluorescence from red (high membrane potential) to green (low membrane potential) with low concentration of CPA, which indicates a loss of membrane potential in the granulosa cell layer. Ovarian follicle viability was also assessed by TB and FDA–PI staining.

Further investigation on the effect of methanol involved the determination of ovarian follicle mtDNA copy number, measurements of ATP and ADP/ATP ratio following cryoprotectant exposure. The effect of Me_2SO was also investigated to determine whether the mitochondrial function decreased further with a CPA known to be more toxic to zebrafish ovarian follicles [14,25,32]. MtDNA copy number, measurements of ATP and ADP/ATP ratio following Me_2SO exposure were also investigated.

Materials and methods

Ovarian follicle collection

Adult female zebrafish (*Danio rerio*) were maintained, handled and sacrificed in line with protocols agreed by the Home Office and the University Research Ethics Committee as meeting all current regulations. Follicles were collected from females euthanatized with a lethal dose of tricaine (0.6 mg/ml) for 5 min and decapitated before ovary removal. The ovaries were immersed in 1.6 mg/ml hyaluronidase [11] in Hanks' solution (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$) for 10 min at room temperature. Follicles were separated by gentle pipetting, washed three times in Hanks' solution, and stage III follicles selected according to morphological appearance as described by Selman et al. [27].

CPA treatment

Follicles were exposed for 30 min at 22 °C to methanol or Me_2SO in Hanks' solution in the concentration range of 1–4 M. Control follicles were incubated in Hanks' solution under the same conditions. For the cryoprotective toxicity test, 15–40 follicles were held in Hanks' solution (10 min) followed by replacement of the medium with CPA supplemented medium. After incubation in CPA for 30 min at 22 °C, follicles were washed twice with Hanks' solution and then held in Hanks' solution at 27 °C. Mitochondrial activity and distribution were assessed for ovarian follicles exposed to methanol within 1 h after the treatment. Assessment of ATP, ADP/ATP ratio, mtDNA copy number were carried out after 1 and 5 h incubation following the CPA (methanol or DMSO) removal. FDA–PI and TB staining tests were carried out after 5 h incubation following methanol or Me_2SO removal.

Staining of mitochondria with JC-1

Stage III ovarian follicles were stained with JC-1 (Sigma) for 30 min after the exposure to different methanol concentrations for 30 min. JC-1 selectively stains mitochondria with low membrane potential green and mitochondria with high membrane potential red. A 1.5 mM stock solution of the dye was prepared according to manufacturer's instructions, the dye was used at a concentration of 5 μM in Hanks' solution at room temperature. Subsequently the follicles were washed three times with Hanks' solution, transferred to a 35 mm glass bottomed dish (WillCo Dish, INTRACEL, Shepreth, Royston, UK) and observed by confocal microscopy.

Confocal microscopy

Stained samples were examined using a Leica TCS-SP/DM IRBE (Leica, Microsystems (UK) Ltd., Milton Keynes, Bucks, UK) confocal microscope equipped with Ar/Kr laser. Active mitochondria distribution was assessed through a series of optical sections. Objectives (20×, 40× and 63× water immersions), pinhole, filters, gain and offset were kept constant throughout the experiments. Laser excitation and emission filters for the labeled dye were as follows: JC-1 FM_{ex} = 488 nm (excitation), (green) λ_{em} = 510/550 nm (emission), (red) λ_{em} = 580/610 nm (emission). Digital images were obtained with Leica TCS software and stored in TIFF format. At least 30 ovarian follicles in each group were assessed in three repeated experiments.

Viability assessment by FDA–PI staining

A stock solution of FDA (Sigma) was prepared by dissolving 5 mg FDA in 1 ml of acetone. The FDA working solution was freshly prepared before use by adding 40 μl of stock to 10 ml of PBS. The Propidium iodide (Sigma) stock solution was made by dissolving 1 mg PI in 50 ml PBS. For FDA–PI staining, 100 μl (2 μg) of FDA working solution and 30 μl (0.6 μg) of PI stock solution were added directly to the follicles. The follicles were stained in the dark for 3 min [15]. The non-polar fluorescein diacetate molecules enter the cell, are hydrolyzed by cellular esterases to produce the polar compound fluorescein. In viable cells, the fluorescein is unable to pass through the intact membrane, accumulating in the cytoplasm of the cell, whilst damaged cells show a distinct loss of fluorescein through the cell membrane. Live cells with intact membranes are distinguished by their ability to exclude the PI that easily penetrates dead or damaged cells, intercalating with DNA and RNA to form a bright red fluorescence. Follicles fluorescing bright green were considered to be viable, while non-viable cells stained bright red. FDA–PI stained follicles were examined with a LEICA DM IL

(Leica, Microsystems (UK) Ltd., Milton Keynes, Bucks, UK) with two filter cubes: I3 (excitation filter: bandpass (BP) 450–490 nm; dichromatic mirror: 510; suppression filter: longpass (LP) 515) and N.2.1 filter excitation filters: BP: 515–560 nm; dichromatic mirror: 580; suppression filter: LP: 590). This filter arrangement did not permit both green and red fluorescing follicles to be seen simultaneously. At least 90 ovarian follicles in each group were assessed in three repeated experiments.

Viability assessment by TB staining

TB was used to assess membrane integrity. Follicles were incubated in 0.2% Trypan blue (Sigma) in Hanks' solution for 3–5 min at room temperature and then washed in Hanks' solution. Unstained follicles were considered viable, while the follicles stained blue were considered non-viable. At least 90 ovarian follicles in each group were assessed in three repeated experiments.

MtDNA copy number determination

MtDNA copy number was measured for follicles after 1 and 5 h incubation following 30 min CPA exposure, holding the follicles in Hanks' solution at 27 °C. Treated and control follicles were stored at −80 °C prior to DNA extraction.

DNA extraction

DNA was extracted from individual ovarian follicles using the EZNA Gel extraction kit (Omega Bio-tek, via VWR, Lutterworth, UK) with a slightly modified protocol. Samples were dissolved in 150 μl binding buffer and vortexed vigorously before being added to the spin columns provided. The remainder of the procedure was carried out according to the manufacturer's instructions.

Generation of standards for real-time PCR

The external standard for mtDNA copy number calculation was generated using conventional PCR with primers designed to amplify a region of the mitochondrial genome (F: TAC AAT CCG CCG CCT AAA CAC T, R: AAG TGC TCC TGG TTG GCT AAG T). Reactions were performed in 20 μl using 1× PCR buffer (Bioline, London, UK), 1.5 mM $MgCl_2$ (Bioline), 0.5 μM of each primer, 200 μM dNTP mix (Bioline) and 2U BioTaq polymerase (Bioline). Reaction conditions were 1 cycle of 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 5 min. PCR products were run on 2% agarose gels and DNA was extracted from the excised bands using the EZNA Gel extraction kit (Omega Bio-tek) according the manufacturer's instructions. Samples were quantified using spectrophotometer at 260 nm, they were initially diluted to 2 ηg/μl and then serially diluted 10-fold for use as standards for real-time PCR.

Real-time PCR

Real-time PCR was performed on a Rotorgene 6000 cycler (Corbett Research, UK) using a 72 well rotor. Reaction tubes contained 7.5 μl SensiMix dT 2× mix and 0.3 μl SYBR Green (both from Quantace), 333 ηM of each primer and 2 μl of each sample or standard, made up to 15 μl with molecular biology grade water. The reaction conditions were 1 cycle at 95 °C for 10 min followed by 50 cycles of 95 °C for 10 s, 59 °C for 15 s and 72 °C for 15 s, with data being acquired on the FAM/SYBR channel at the end of each extension step. Melt curves were analysed to check for the absence of mispriming. Each experiment was carried out three times and all samples were run in triplicate. The concentration of each standard was converted into mtDNA copy number and the number of mtDNA molecules per follicle for each treatment was subsequently calculated using the RotorGene software (Version 1.7, Corbett Research) and Microsoft Excel.

ATP levels and ADP/ATP ratio determination

Calculation of ADP/ATP ratio provides more information on mitochondrial energetic status than measurement of the ATP content alone. ADP/ATP ratio and ATP levels were evaluated in follicles after 1 and 5 h incubation following CPA removal, the ovarian follicles were held in Hanks' solution at 27 °C. Follicles from control groups and follicles exposed to 1–4 M methanol or to 1–4 M DMSO for 30 min, were used for the preparation of extracts for determination of ATP + ADP levels. For all experiments, three replicates were used for each treatment and experiments were repeated three times. For the extract preparation the procedure described by Guan et al. [12] was used: 30 stage III zebrafish ovarian follicles were added to 1 ml of ice cold 0.5 M perchloric acid and 4 mM EDTA and homogenized with a conical glass pestle. The homogenate was centrifuged at 20,000*g* for 5 min at 0–2 °C in refrigerated centrifuge. The supernatant was separated and neutralised to between pH 7 and 6 with 2.5 M KOH. The neutralised supernatant was centrifuged for 5 min at 8000*g* and the new supernatant collected. This extract was loaded into an eppendorf tube and stored at −20 °C until required.

ATP content of ovarian follicle extracts was measured using the luciferin–luciferase bioluminescence assay provided by a commercial kit (ApoSENSOR™ ADP/ATP Ratio Assay Kit, BioVision). A luminometer (TD-20/20 – Turner Designs) was used for all measurements. Background light was measured and subtracted by running a blank containing water. The measurements were expressed as the number of relative light units (RLU). The nucleotides were released from the extract by addition of 100 μl nucleotide releasing reagent. About 1 μl of ATP monitoring enzyme was added to the lysate and a reading was taken at 1 min to determine the ATP level (A). To measure the ADP levels in the cells, the samples were read at 10 min (B). The ADP in the wells was converted to ATP by the addition of 1 μl of ADP converting enzyme and a reading was taken after 1 min (C).

The ratio of ADP:ATP for each well was calculated from these three readings as follows:

$$\frac{C - B}{A} = \frac{ADP}{ATP}$$

The mean and standard error of the triplicates were calculated.

Statistical analysis

Statistical analysis was carried out using SPSS (SPSS for Windows Version 12.0) and Microsoft Excel. The one-sample Kolmogorov–Smirnov test was performed to determine whether the data were normally distributed. As all data were normally distributed, differences in mtDNA copy number between each treatment and the relevant control group were calculated using *t*-tests. For ATP data analysis, FDA–PI and TB results, comparisons among groups were made by one-way ANOVA. Where differences were found Tukey's post hoc test was carried out to establish which samples were different. All data were expressed as mean ± SEM across the three replicates and *P* values of less than 0.05 were considered to be significant.

Results

Effect of methanol assessed by confocal microscopy

We have previously demonstrated that methanol was a relatively non-toxic CPA, the No Observed Effect Concentrations (NOECs) for stage III ovarian follicle was >3 M assessed by GVBD, TB and FDA–PI tests that methanol was a relatively non-toxic CPA [32]. In

order to determine whether this CPA had any effect on mitochondria, JC-1 staining was carried out. This allowed observation of potential changes in distribution, network formation and membrane potential ($\Delta\Psi$). The results obtained using confocal microscopy after exposure of zebrafish stage III ovarian follicles to methanol, showed that even low concentrations of methanol induced changes in mitochondrial membrane potentials in the granulosa cells which surrounded the oocyte, resulting in a loss of red fluorescence and decrease of green fluorescence (Fig. 1A1, B1, A2, B2, A3, B3, A4 and B4) compared with control group (Fig. 1A0, B0). Red fluorescence, an indicator of mitochondria with high membrane potential, decreased with 2 M methanol, whilst higher concentrations, 3 or 4 M, also caused a loss of the mitochondrial distribution pattern (Fig. 1A3, B3, A4 and B4).

Viability assessed by TB and FDA–PI staining

In order to determine the toxicity of methanol and Me_2SO, two viability assessments were carried out at 1 and 5 h after treatment. The viability by TB and FDA–PI staining at 1 h is not presented here as these data have been previously published [32]. No Observed Effect Concentrations (NOECs) for stage III ovarian follicle were >3 and 1 M for methanol and Me_2SO respectively [32].

There was a decrease of viability of the control groups following 5 h incubation at 27 °C in Hanks' solution when the viability was assessed by both TB and FDA–PI. However, there were no significant differences ($P > 0.05$) between the control group at 5 h post-treatment and the equivalent groups treated with 1–4 M methanol when the viability was assessed by TB. When FDA–PI was used, there were significant decreases in viability after 5 h incubation following 30 min exposure to 3 or 4 M methanol (Fig. 2A).

There were no significant differences between the control group after 5 h incubation and the groups treated with 1 M Me_2SO when the viability was assessed by TB. When assessed using FDA–PI staining, there were significant decreases in viability after 5 h incubation following Me_2SO removal (Fig. 2B). Methanol was the least toxic cryoprotectant to zebrafish ovarian follicles only showing significant decreases in viability after 5 h incubation following 30 min exposure to 3 or 4 M methanol when assessed by FDA–PI. However, DMSO was more toxic to stage III follicles as it significantly reduced the viability of follicles when assessed both by FDA–PI or TB (Fig. 2B).

Effect of cryoprotectants on mtDNA copy number

As mtDNA copy number and mitochondrial number are reported to correlate to potential activity [30], mtDNA copy number was measured to enable quantification of the effect of cryoprotectant incubation. Results showed that there were no significant differences ($P > 0.05$) between the control and treated follicles when the mtDNA copy number was evaluated 1 h after the 30 min exposure to methanol (Fig. 3A). However, there were significant ($P < 0.05$) decreases in mtDNA copy number between untreated follicles and those exposed to concentrations of 3 or 4 M methanol when the mtDNA copy number was evaluated 5 h following the removal of the cryoprotectant (Fig. 3A). Furthermore, there were significant differences between the groups assessed for mtDNA copy number after 1 h incubation and those measured after 5 h incubation following methanol treatment when 2, 3 or 4 M was used, which was not the case for control samples as there were not significant differences between the control groups incubated for 1 h and those incubated for 5 h in Hanks' solution. As effects were observed with relatively non-toxic methanol, the effect of Me_2SO was investigated, which is known to be more toxic than methanol, but still a commonly used CPA.

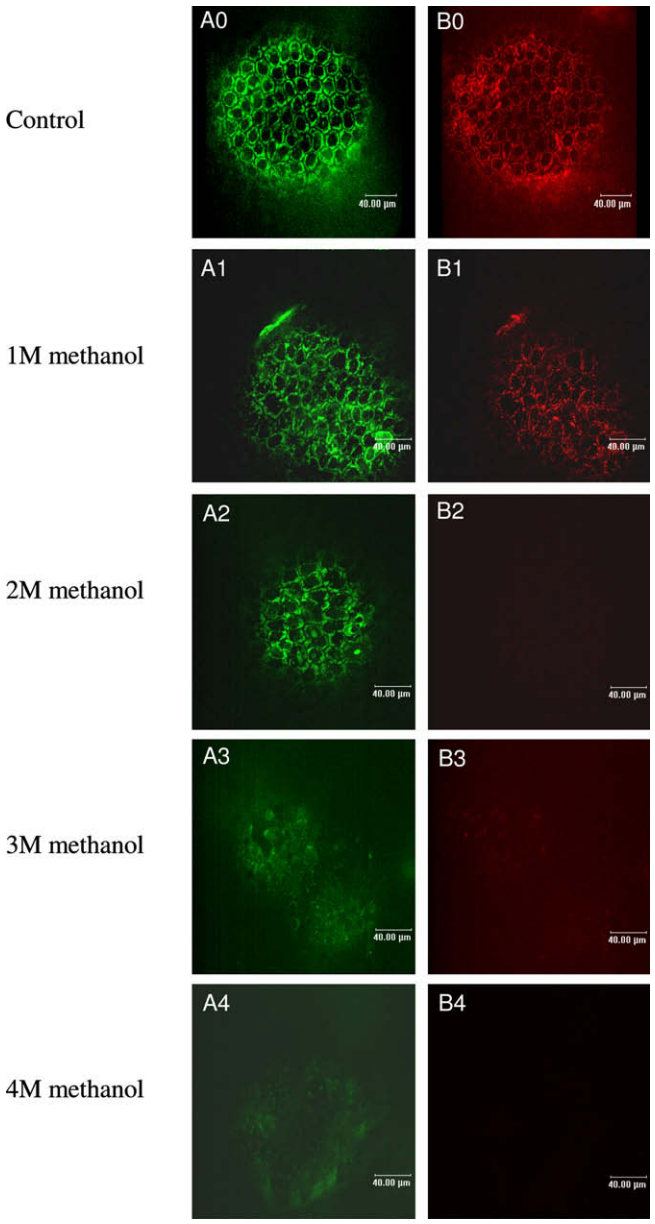

Fig. 1. Effect of different concentrations of methanol on mitochondrial membrane potential and mitochondrial distribution in the granulosa cells of stage III zebrafish follicle. Ovarian follicles were exposed to JC-1 immediately after 30 min treatment with CPA. JC-1 is a sensitive marker for mitochondrial membrane potential ($\Delta\Psi$m). JC-1 accumulates in monomeric form within the mitochondrial matrix and its fluorescence emission characteristics are a function of the magnitude of $\Delta\Psi$m. Low polarized organelles fluoresce green, while higher polarized organelles fluoresce red owing to multimerisation of JC-1 and formation of J-aggregates. The control shows mitochondria with low membrane potential stained green (A0) and mitochondria with high membrane potential stained red (B0). 1 M methanol causes a decrease in both green (A1) and red (B1) fluorescence. 2 M methanol also causes a decrease in green fluorescence (A2) and loss of red fluorescence (B2). 3 M (3A, 3B) and 4 M methanol (4A, 4B) cause a structural breakdown of the mitochondrial patterns.

An increase in mtDNA copy number was obtained between 1 and 5 h in control samples and for 1 M Me_2SO. Such increase was not present in samples treated with 2, 3 and 4 M Me_2SO. There was a significant early increase ($P > 0.05$) between the untreated ovarian follicles and those exposed to 2 M Me_2SO when the mtDNA copy number was measured 1 h after the CPA removal (Fig. 3B). Significant decreases ($P < 0.05$) were found between the untreated ovarian follicles and those exposed to 3 and 4 M Me_2SO when the

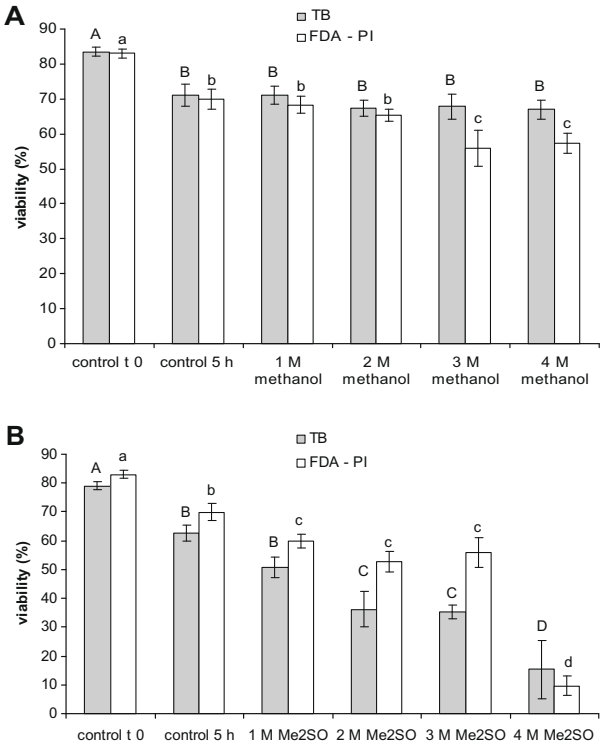

Fig. 2. Viability assessed by TB and FDA–PI staining after exposure to methanol (A) or Me_2SO (B) followed by incubation in Hanks' solution for 5 h at 28 °C. Bars and error bars represent means ± SEM of three experiments each with three replicates. Bars with different superscripts differ significantly ($P < 0.05$).

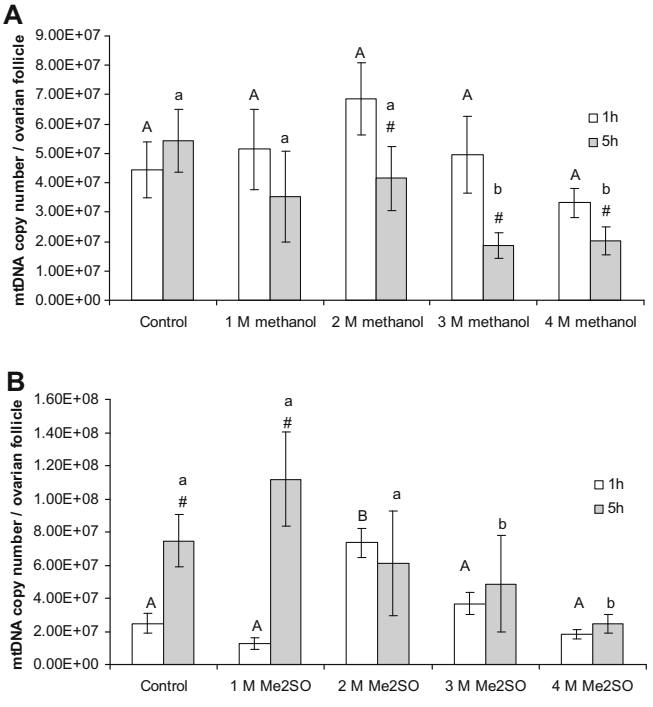

Fig. 3. Effect of methanol (A) and Me_2SO (B) exposure on mtDNA on zebrafish stage III ovarian follicles. mtDNA copy number was measured 1 and 5 h after 30 min exposure to cryoprotectant. Error bars represent standard error of the mean. Different letters represent significant differences between control and treated groups. ($P < 0.05$). # represents significant differences between the 1 and 5 h pair values.

mtDNA copy number was measured after 5 h incubation following the CPA removal (Fig. 3B).

Effect of cryoprotectants on ATP content and ADP/ATP ratio

In order to determine whether the changes observed in mitochondrial distribution, membrane potential and mtDNA copy number actually affected mitochondrial function, ATP levels and ADP/ATP ratios were determined.

Results from these studies showed that the ATP content was not significantly affected after 1 or 5 h incubation following 30 min exposure to 1–4 M methanol (Fig. 4A), there were no significant differences between the control group and the treated groups ($P > 0.05$). Similarly, at 1 h following 30 min exposure to methanol there were no significant differences between control groups and treated groups when the ADP/ATP ratio was calculated. However, all the treated groups had lower ADP/ATP ratios then the control groups at 5 h following methanol removal, although the differences were not statistically significant (Fig. 4B).

The results also showed that ATP content significantly decreased ($P < 0.05$) following exposure to 2, 3 and 4 M Me_2SO when measured both at 1 and 5 h following CPA exposure for 30 min (Fig. 4C). At 1 h following Me_2SO removal the ADP/ATP ratio decreased compared to the control group when 2 and 3 M were used, whilst at 5 h post-treatment there was a decrease of ADP/ATP ratio for all the treated groups (Fig. 4D), however the differences were not statistically significant.

Discussion

Cryoprotectants are essential for the successful freezing and thawing of viable cells but are known to be toxic at high concentrations. The results obtained from viability tests previously performed in zebrafish ovarian follicles [25,32] and from the present study showed the relatively low toxicity of methanol at concentrations of 3 or 4 M, when the viability was assessed based on membrane integrity. These results could be due to the fact that cell membranes are generally highly permeable to methanol and that the methanol does not induce any damage in terms of membrane integrity. Zhang et al. [34] reported that methanol penetrates ovarian follicles at a rate comparable with the rate of water transport and therefore, incubation of cells with this cryoprotectant does not lead to osmotic stress.

Our hypothesis was that even cells that appear unaffected by freezing or CPA exposure may suffer more subtle effects. In support of this, our data obtained by JC-1 staining with confocal microscopy indicate a reduced fluorescence and an increasing loss of the well organised distributional arrangement with increasing concentration of methanol. Images obtained by confocal microscopy showed the disruptive effect of methanol on mitochondrial distribution and mitochondrial membrane potential in the granulosa cells, which surround the stage III zebrafish oocyte. These findings showed that methanol exposure, even at concentrations that did not affect survival, resulted in a loss of membrane potential. JC-1 has been used to estimate variation in membrane potential. It has been shown in mammalian cells that mitochondrial membrane potential is a key indicator of cellular viability [1,20], as it reflects the pumping of hydrogen ions across the inner membrane during the process of electron transport and oxidative phosphorylation, responsible for ATP production. Many mitochondrial functions, including protein import, ATP generation and lipid biogenesis, depend on the maintenance of $\Delta\Psi m$ [31]. JC-1 proved to be a useful marker of mitochondrial activity and mitochondrial distribution arrangement.

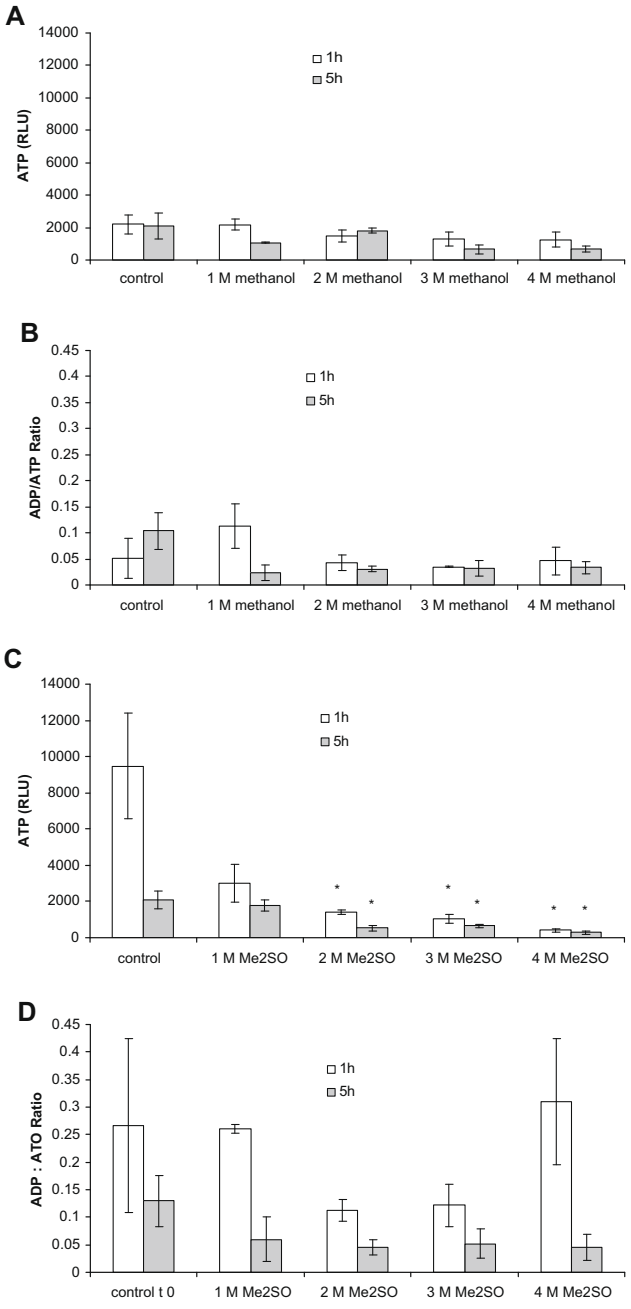

Fig. 4. Effect of methanol (A–B) and Me_2SO (C–D) exposure on ATP level and ADP/ATP ratio on zebrafish stage III ovarian follicles. ATP level and ADP/ATP ratio were measured 1 and 5 h after the exposure to cryoprotectant for 30 min. Error bars represent standard error of the mean. Significant difference in ATP level between control and treated groups are indicated ( ) ($P < 0.05$).

The role of granulosa/thecal cells has already been established in supporting oogenesis, vitellogenesis and maturation [4,8]. Also the mitochondria in the granulosa layer have different functions not only as a source of ATP, but also as a source of cAMP and in the synthesis of steroids. Therefore, a compromised mitochondrial activity might result in failure of oocyte maturation. In these experiments it was not possible to observe mitochondria in the oocyte itself due to the thickness of the vitelline envelope and the consequent failure of the mitochondrial probe to penetrate the oocyte. Should the methanol reach the oocyte and compromise the mitochondria, its impact may severely affect the success of *in vitro* maturation and later embryo development, taking into consideration the critical role that mitochondria, which are maternally inherited organelles, have during early embryonic development.

It has previously been reported that cryopreservation of zebrafish (*Danio rerio*) blastomeres [16] increases the frequency of mtDNA mutations. In order to see if CPA treatment could also affect mtDNA and to quantify the effects seen with methanol exposure, we measured mtDNA copy number.

At 1 h after exposure to methanol for 30 min no changes in mtDNA copy number were obtained despite structural damage observed by confocal microscopy. A decrease of copy number was obtained at 5 h after the exposure to 3 and 4 M methanol for 30 min. About 3 and 4 M methanol also resulted in a decrease in viability assessed by FDA–PI staining. The decrease in copy number could be due to a compromised synthesis mechanism of mtDNA following methanol exposure.

Exposure to 2 M Me_2SO resulted in a marked increase in mtDNA copy number 1 h after 30 min exposure. This increase could be an attempt by the cells to correct for the relatively mild insult. At 3 and 4 M Me_2SO, there was no increase in mtDNA copy number, possibly due to the synthesis mechanism being damaged beyond repair. This may have contributed to the decrease of viability observed in these ovarian follicles when assessed by both TB and FDA–PI tests at 1 and 5 h after the removal of CPA.

MtDNA encodes vital components of the electron transport chain, essential for efficient production of cellular ATP. It is also known that the impact of depletion of cellular ATP in oocytes induces dramatic disruption of microfilaments, as well as cessation of the many other vitally important processes [17].

The aim of the ATP content and ADP/ATP ratio studies was to determine whether the changes in mtDNA copy number were also reflected in changes in mitochondrial function on the ovarian follicle. At 1 h after exposure, ATP content decreased with increasing concentration of methanol. At 5 h after exposure, there was still a trend towards decreased ATP in treated samples but it did not correlate with concentration. The ADP/ATP ratio showed a similar trend as the ATP content but in this case, a larger decrease was noted after 5 h from the treatment. While necrotic cell death may be associated with an early decrease in the ability of the cells to synthesize ATP, cells undergoing apoptosis may retain a high concentration of ATP until relatively late in the progression of events leading to cell death [6,18]. With the exception of 1 M Me_2SO, the exposure to Me_2SO induced a decrease of ATP content and the ADP/ATP ratio showed a trend of decrease 5 h after CPA removal; whilst at 1 h a decrease was only observed with 2 and 3 M Me_2SO. These results could indicate that most of the intracellular ATP and probably ADP stocks have been consumed in coping with stress associated with cryoprotectant exposure, or an irreversible damage of the cell vital energy system. The results from methanol exposure together with the results obtained measuring the ADP/ATP ratio after Me_2SO exposure suggested a very large variation in ATP content among ovarian follicles. Perhaps the influence of this variation could be minimized by increasing the size of the sample. In the present work, 30 ovarian follicles were used per group. Although effects were not large enough to show statistical difference, the observation of similar trends between treatments and between copy number, ATP content and ADP/ATP ratio suggests that there are biological effects present.

In this study, the effect of methanol exposure on mitochondrial activity and distribution was investigated by confocal microscopy. Exposure to 2 M methanol induced a loss of membrane potential showed by JC-1 stain, although viability tests showed no decrease in survival even after 5 h incubation following CPA removal. Higher concentrations of methanol (3 and 4 M) induced not only a decrease of mitochondrial membrane potential but also loss of mitochondrial contiguous distribution at the margin of each granulosa cell [33]. Furthermore 3 and 4 M treatments resulted in a decrease

of viability assessed by FDA–PI after 5 h incubation following CPA removal, whilst a decrease in mtDNA copy number and ADP/ATP ratio occurred after 5 h incubation following 30 min exposure to methanol indicating a delayed effect.

Exposure to Me_2SO (2, 3 and 4 M) induced an immediate and sustained decrease of ATP and accompanied by failure to maintain mtDNA copy and also by a decrease of survival rates already after 1 h incubation following CPA removal.

The effect of cryoprotectants on mitochondria of zebrafish ovarian follicles is reported here for the first time. The impact of cryoprotectants at the sub-cellular level will inform the development of optimal cryoprotocols, especially if key indicators can be identified that would allow early detection of adverse effects. In this current study, the effects of cryoprotectants on five mitochondria related properties were investigated – mitochondrial membrane potential, mitochondrial distribution, mtDNA copy number, ATP levels and ADP/ATP ratios. We detected adverse effects at relatively low concentrations of methanol, previously described as the least toxic of the cryoprotectants used with zebrafish oocytes and embryos. The first impact of cryoprotectants was a decrease in membrane potential, as seen with JC-1 fluorescence staining. This in turn led to a cascade of other effects, namely loss of the hexagonal distribution pattern of mitochondria in the granulosa cells and a reduction in total follicular ATP levels and mtDNA copy number.

The development of an accurate and rapid assay to detect changes in mitochondrial membrane potential during cryoprotocols would be a valuable tool in protocol design. This is an area of work we are now undertaking.

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