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Title:

SHORT TERM CHILLED STORAGE OF ZEBRAFISH (Danio *rerio*) EMBRYOS IN CRYOPROTECTANT AS AN ALTERNATIVE TO CRYOPRESERVATION

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Abstract

As zebrafish embryos never been cryopreserved, we developed a protocol to store zebrafish embryos (50% epiboly- 5.3 hpf) for up to 18 h at 0° C. Initial experiments to optimise cryoprotectant solution demonstrated improved embryo hatching rate following chilling at 0°C for 18 h with 1 M MeOH + 0.1 M sucrose (56 ± 5%) compared to other combination of methanol (0.2-, 0.5 M) and sucrose (0.05-, 0.1 M). This combination of cryoprotectants that protects against chilling injury was further tested to assess its impact on *sox* gene and protein expression. Significant decreases in *sox3* gene expression were observed in hatched embryos that had been chilled for 18 h in 1 M MeOH + 0.1 sucrose compared to non-chilled controls, however expression of both *sox2* and *sox3* proteins was unaffected. Significant decreases in *sox2* protein expression were however observed in embryos that had been chilled without cryoprotectants and these embryos also had lower hatching rates than those chilled with the optimal cryoprotectant solution. We therefore conclude that the cryoprotectant combination of 1 M MeOH + 0.1 M sucrose facilitates chilled storage of early stage (50% epiboly) zebrafish embryos for up to 18 h without compromising transcriptional response.

1 Introduction

Chilled storage of zebrafish embryos is becoming increasingly important due to the fact that cryopreservation of fish embryos is still a challenge due to their structural limitations [1]. Storage of aquatic embryos at low temperatures for extended periods has important applications in aquaculture and fish farming. Chilled storage has practical applications in fish embryo handling e.g. for embryo transportation between fish farms for genetic improvement programs. However fish embryos of several species including zebrafish have been shown to be chilling sensitive [2-5]. Zhang and Rawson [3] showed that early stage zebrafish embryos were more chilling sensitive than later stages but the methanol solution containing sucrose in an enhanced survival of heartbeat stage embryos was achieved following chilled storage for 18 and 24 h at 0°C. Higher survival had also been seen in common carp (*Cyprinus carpio L.*) embryos 12 hour post fertilization (hpf) when they were chilled at zero and sub-zero temperature for 12-72 h in presence of MeOH (1.5 M) and sucrose (0.5 M) compare to using these cryoprotectants (CPA) separately or without CPAs [6]. However, all these studies are based on the results obtained on embryo hatching/survival rate and did not provide information on molecular stability i.e. gene and protein expression. It has been shown that storage of zebrafish embryos using propylene glycol in the liquid nitrogen even for 1 min resulted in damage of mitochondria, disorganisation of ribosomes and plasma membrane of the yolk syncytial layer [7]. In our previous studies [8], significant decreases in *sox2* (up to 4-fold) and *sox3* (up to 3-fold) were observed in embryos that had been chilled for up to 180 min at 0 ° C. Therefore information on the effect of chilling and cryopreservation on embryos at the molecular level is important in designing protocols for successful chilled storage and cryopreservation of the embryos.

In recent years, zebrafish has become an important animal model in scientific research. Zebrafish is used extensively for developmental biology studies such as determination of the embryonic axis [9], cell lineage analysis [10], formation of the central and peripheral nervous system [11], cardiovascular development [12] and differential regulation of gene expression [13]. Studies have also revealed a close relationship between the zebrafish and human genomes and it has been proposed that zebrafish could be a bridge between study of human disease and development due to the significant homology

between zebrafish and human genomes [14]. In the current study, *sox* genes have been used as a marker, due to their multi-functional roles during development [15-17] to evaluate the effect of long term chilling on gene and protein expression.

The aim of the present study was to enhance hatching rates of early stage zebrafish embryos by identifying conditions that do not have an impact on *sox* gene expression. Studies were carried out to investigate the effect of chilling and warming on gene and protein expression in the embryos that had been chilled with cryoprotectant mixtures for up to 18 h and warmed and cultured until the hatching stages.

2 Materials and methods

Zebrafish maintenance and embryo selection

Adult zebrafish 12-14 weeks old were maintained in 40 litre glass tanks at 28° C. The males and females were kept at a ratio of 1:2 and a 12 hour light/dark cycle was used. Fish were fed three times a day with TetraMin® (Tetra, Germany) flake food and once a day with freshly hatched brine shrimp (*Artemia salini*) (ZM systems, UK). Embryos were collected in the morning and kept in a 28° C water bath until the desired stage was reached. Embryonic stages were determined using light microscopy (Leica MZ95, Germany) according to the morphology described by Kimmel [18].

Experimental Design

Experiment 1: Assessing the effect of 18 h chilling on hatching rate in zebrafish embryos in the presence of sucrose and MeOH mixture

Embryos (50% epiboly) were chilled at 0°C for 18 h with different concentrations of MeOH (0.2 M, 0.5 M and 1 M) in combination with sucrose (0.05 M, or 0.1 M). Embryos (20 embryos/ 3mL cryoprotectant) were chilled at 0°C in a crushed ice bath for 18 h in test tubes and the temperature was maintained using crushed ice [19]. After chilling, the cryoprotectant solution was replaced with Embryonic medium 2 (EM2, 15 mM NaCl, 0.5 mM KCl, 0.27 mM $CaCl_2$, 1 mM $MgSO_4$, 0.27 mM $NaHCO_3$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4) and the test tubes were quickly placed into a

27±1°C water bath. Embryos were washed three times with 27±1°C EM2 and then incubated at 27±1°C until they hatched. Control embryos were kept at 27±1°C throughout. Hatching rates were then calculated and all experiments were performed three times (20 embryo/treatment) in triplicate using total of 1260 embryos. Embryo hatching rates were examined at 72 h post treatment according to morphological changes described by Kimmel [18]. Embryos were considered to be hatched when their chorion was missing, there were no obvious signs of malformation, and there was natural movement with a functional heartbeat. Embryos were considered unhatched and removed if they showed no sign of cell differentiation, yolk coagulation, lacked tail formation, had detached tails and/or if they remained in the chorion [20].

Experiment 2: Assessing the effect of 18 h chilling on *sox* gene expression in zebrafish embryos

The optimal cryoprotectant mixture 1 M MeOH + 0.1 was identified and used in this study to investigate the effect of chilling on embryo gene expression. Embryos at 50% epiboly stage were chilled at 0° C for 18 h in the optimal cryoprotectant mixture (1 M MeOH + 0.1 M Sucrose) as described in experiment 1. RNA from each treatment category (0 h, 18 h chilled, 18 h control and 18 h chilled in cryoprotectant mixture and control at 27 °C) was then extracted and cDNA was produced and diluted at a ratio of 1:2 with molecular biology grade water (Sigma, UK) for use in real time PCR, as described below. Each experiment was repeated three times, with three groups of five embryos per treatment category being used for each replicate (n = 225).

Experiment 3: Assessing the effect of 18 h chilling and warming on *sox* (*sox2, sox3* & *sox19a*) gene expression in zebrafish embryos

This experiment was performed in order to identify the *sox2,sox3* and *sox19a* gene expression patterns in chilled embryos after warming and subsequent incubation upto the hatching stage. 50% epiboly staged embryos were chilled in the optimal cryoprotectant mixture (1 M MeOH + 0.1 M sucrose) as described in experiment 1 and warmed at 27±1°C. They were then incubated at 27 ± 1°C until they reached the hatching stages. RNA from different stages [before chilling (0 h), after chilling (18 h), 20

somites, heartbeat and hatching] was extracted and cDNA was produced as per experiment 2. cDNA was diluted at ratio of 1:2 with molecular biology grade water (Sigma, UK) for use in real time PCR. For each stage, three different biological samples (5 embryos/tube) were used. Each experiment was repeated three times (n=225). Experimental controls were kept at 27±1°C ° in a water bath for the equivalent time period.

Experiment 4: Assessing the effect of 18 h chilling and warming on *sox2* and *sox3* protein expression in zebrafish embryos

In order to further understand whether the gene expression patterns observed in experiment 3 had an impact on translation. Protein expression studies were carried out for *sox2* and *sox3* following 18 h chilling and after hatching. Chilling and warming was carried out as described in experiment 3. Following treatment, proteins were extracted immediately from 75 embryos per treatment category (0 h , 18 h RT , 18 h chilled, 18 h chilled with cryoprotectant solution – for 18 h chilled time points) and hatching stages (18 h RT, 18 h chilled-warmed, 18 h chilled with cryoprotectant solution and warmed – for 18 h chilled and warmed time points) as described below and stored at -80°C.

RNA extraction and DNase treatment

RNA was extracted from embryo samples using RNAqueous Micro RNA Isolation Kit (Ambion, UK) according to the manufacturer‘s protocol. This protocol also includes a DNase I treatment step. RNA was stored at -80 ° C until further use and was checked for quantity and purity using a Biophotometer (Eppendorf, UK) at 260 nm and 280 nm.

Reverse transcription

RNA (1 µg) was transcribed using Precision qScript Reverse Transcription Kit (Primerdesign Ltd, UK) according to the manufacturer’s protocol. For conventional PCR, undiluted cDNA was used in subsequent steps. For real time PCR experiments, cDNA was diluted 1:2 in molecular biology grade water (Sigma, UK) and stored at -80° C.

Conventional PCR Analysis

The PCR reactions consisted of NH_4 PCR buffer (Bioline, UK), 200 µM dNTP (Bioline), 1.5 mM $MgCl_2$ (Bioline), 2 U BIOTAQ™ DNA polymerase (Bioline), 0.5 µM each primer (see Table 1), 1 µg RNA template and PCR water up to a value of x. Standard conditions for PCR were initial denaturation at 94° C for 5 min, 40 cycles of 94° C for 30 sec , annealing temperature (see Table 1) for 30 sec and 72° C for 30 sec, followed by 10 min additional extension at 72 °C. The PCR products were run on 2% agarose gels and stained with ethidium bromide (0.5 µg/mL, Sigma, UK).

Generation of standards for real time PCR

The standards for real time PCR of *sox2*, *sox3* and *sox19a* along with housekeeping genes EF1-α and β actin [21] were produced using conventional PCR as described above. The primer sequences are given in the Table 1. DNA was isolated from excised gel bands using the EZNA Gel extraction kit (Omega Bio-Tek through VWR, UK) according to the manufacturer's instructions. The isolated DNA was quantified using a Biophotometer (Eppendorf, UK) at 260 nm and diluted to 2 ng/µl followed by 10-fold serial dilutions to generate standards for real time PCR.

Quantification of *sox2*, *sox3* and *sox19a* using real time PCR

Real time PCR was performed on a RotorGene 6000 cycler (Corbett Research, UK) using a 72 well rotor to quantify the expression levels of *sox2*, *sox3* and *sox19a*. Reaction tubes contained 7.5 µl of sensimix 2X reaction buffer (containing heat activated DNA polymerase, Ultrapure dNTPs, $MgCl_2$, SYBR® Green I), 333 ηm of each primer (see Table 1) and 2 µl of cDNA sample, made up to 15 µl with PCR water. The reaction conditions were 95° C for 10 min, 50 cycles of 95° C for 10 sec, the appropriate annealing temperature (see Table 1) for 15 sec and 72° C for 15 sec. Data were acquired on the FAM/SYBR channel at the end of each extension step. Melt curves were analysed to check for the absence of mispriming and amplification efficiency was calculated from a standard curve (efficiencies ranged from 0.8 to 1.0 and R^2 values from 0.99 to 1). The possibility of genomic DNA amplification was eliminated by use of primers that crossed introns. Relative gene expression levels were calculated using the two standard curve quantification method in the Rotorgene software [22]. Ef1

α and β actin were used as housekeeping genes (HKG) for this study as these genes were previously shown to have the highest stability during chilling of zebrafish embryos [21].

Protein expression analysis

Extraction of protein and quantification: Embryos (75 embryos/treatment) were washed twice with EM2. Before being subjected to protease treatment (2 mg/ml, Sigma-Aldrich) for 10 min to partially digest the chorions. Loosened chorions were than removed by gentle suction and friction, resulting from pipetting the embryos up and down. Dechorionated embryos were washed a further three times with EM2 before being transferred to a 1.5 ml tube. 100 µl of protein extraction buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol) was added to each tube and samples were heated to 95°C for 10 min. Following heating, samples were vortexed and centrifuged at 13,000 x g for 10 min and protein containing supernatants were collected. Isolated proteins were quantified using the QuantiPro™ BCA Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions.

Western blot and immunostaining: Extracted protein was separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins (40 µg) were loaded on 4% stacking gel and separated on 10% resolving gels at 200 V for 40-60 min. Proteins were then transferred to PVDF membranes using the semi dry method (BioRad, UK). Immunoblotting was carried out using an Anti rabbit WesternDot 625 Western Blot Kit (Invitrogen, W10132). The membrane was blocked in 10 ml WesternDot blocking buffer for 1 hour at room temperature. Following blocking, membranes were incubated with 10 ml primary antibody solution (dilution 1:1000) overnight at 4˚C on a gel rocker. Primary antibodies against *sox2* (Abcam, Cambridge, UK), *sox3* (Eurogentech, Belgium) and *β-actin* (Eurogentech, Belgium) were used at 1:1000 concentration diluted in PBS. The membranes were then washed 3 times for 10 min with WesternDot Wash buffer. Following washing, the membranes were incubated with 10 ml Biotin-XX-Goat anti-rabbit solution (1:10000) for 2 hours at room temperature. After secondary antibody incubation, the membranes were further washed 3 times. The membranes were then incubated with 10 ml Qdot 625 Streptavidin conjugate solution for 1 hour at room

temperature before being washed 3 times as previously described, followed by a final wash in MilliQ water for 5 min. The membranes were soaked in 100% methanol to make them transparent and then visualised under a UV trans-illuminator.

Statistical Analysis

Statistical analysis was carried out using SPSS V.16 (IBM, USA) and Microsoft Excel (Microsoft corp. USA). Densitometry analysis was carried out using ImageJ software (Maryland, USA). All protein bands were quantified and then normalised with respect to non-treated samples. The one-sample Kolmogorov-Smirnov test was performed to determine whether the data for each gene/protein were normally distributed. Where the data were normally distributed, significant differences in gene/protein expression levels between fresh and chilled embryos at the same time point were calculated using a t-test and the one way ANOVA was carried out followed by Tukey's post hoc tests to identify changes in gene/protein expression levels between treatments. Where data were not normally distributed after logarithmic transformation, the Mann-Whitney U test was used instead. All gene and protein expression data were presented as mean ± SEM and p values of less than 0.05 were considered significant.

3. Results

Experiment 1: The effect of 18 h chilling on hatching rate in zebrafish embryos in the presence of sucrose and MeOH

Significant increases ($p < 0.05$, Tukey's post hoc test) in hatching rates were observed in the embryos that were chilled with 0.5 and 1 M MeOH + 0.1 M sucrose mixtures (45 ± 10% and 56 ±5%) when compared to embryos that had been chilled in different MeOH concentrations with 0.05 M sucrose [1 M MeOH + 0.05 sucrose (15± 5 %), 0.5 M MeOH + 0.05 sucrose (20 ± 5%), 0.2 M MeOH + 0.05 sucrose (10 ± 3%) and 0.2 M MeOH + 0.1 M sucrose (20 ± 4%)] (Fig 1). No significant differences in hatching rates were observed among embryos treated in 0.05 M sucrose with different concentrations of MeOH.

Experiment 2: The effect of 18 h chilling on *sox* gene expression in zebrafish embryos

In non-chilled control embryos after 18 h at 27± 1℃, *sox2* (Fig 2a) gene expression increased significantly ($p < 0.05$, Tukey's post hoc test) when compared to time zero (0 h). However in the embryos that were chilled at 0℃ for 18 h without cryoprotectant, *sox2* gene expression was significantly lower than non-chilled controls (Fig 2). No significant differences were observed between non chilled embryos and those that had been chilled for 18 h in the cryoprotectant mixture containing 1 M MeOH and 0.1 M sucrose. *Sox3* (Fig 2b) gene expression decreased significantly ($p < 0.05$, Tukey's Post hoc test with log transformation)- after 18 h of development at RT and no significant differences were observed in chilled embryos with or without cryoprotectant when compared to non-chilled 18 h controls. A significant decrease in *sox19a* expression was also observed in 18 h non chilled control embryos when compared to 0 h controls (Fig 2c). However, embryos that had been chilled for 18 h in cryoprotectant mixture containing 1 M MeOH and 0.1 M sucrose had significantly increased *sox19a* expression compared to the non chilled controls.

Experiment 3: The effect of 18 h chilling and warming on *sox* gene expression in zebrafish embryos

In control embryos, gene expression of *sox2* remained stable and *sox3* and *sox19a* (Fig 3) gradually decreased throughout development from 50% epiboly to hatching stage ($p < 0.05$). In the embryos that had been chilled with 1 M MeOH + 0.1 M sucrose for 18 h and then warmed before being cultured to the hatching stage, *sox2* (Fig 3a) and *sox19a* (Fig 3c) gene expression increased just after the chilling period and again increased at the heartbeat stage before levelling up with non-chilled control embryos at the hatching stage. The opposite patterns were observed for *sox3* (Fig 3b) as gene expression decreased significantly ($p < 0.05$, Mann-Whitney U test) when compared to controls at 20 somites stage and recovered at the heartbeat stage before decreasing significantly again at the hatching stage.

Experiment 4: The effect of 18 h chilling and warming on *sox2* and *sox3* protein expression in zebrafish embryos

The effect of 18 h chilling on *sox2* and *sox3* protein expression in 50% embryos chilled with or without 1 M MeOH + 0.1 M sucrose is shown in Fig 4. Protein expression profile is shown in Fig 4(a) along with internal control β actin. Based on these results, densitometry was performed using ImageJ software and data are presented in Fig 4(b). *Sox2* protein expression increased significantly ($p < 0.05$) from 0 h to 18 h in control non chilled embryos. Embryos chilled without cryoprotectants demonstrated significantly decreased *sox2* levels when compared to non-chilled 18 h controls ($p < 0.01$). However, embryos that had been chilled with 1 M MeOH + 0.1 M sucrose demonstrated no significant changes in *sox2* protein expression when compared to 18 h non chilled embryos. *Sox3* protein expression increased significantly in non-chilled 18 h embryos compare to 0 h. No significant differences in *sox3* protein expression were observed in embryos that had been chilled with/without cryoprotectant mixture compare to non-chilled 18 h control embryos.

Equivalent data for these embryos following culture to the hatching stage is shown in Fig 5. *Sox2* protein expression was stable throughout the culture period and no significant differences were observed between non-chilled control embryos and chilled embryos either with/without cryoprotectants. *Sox3* protein expression increased significantly from 0 h (50% epiboly) to the hatching stages in non chilled embryos, chilled embryos with or without cryoprotectant.

4. Discussion

Chilled storage is an important alternative to cryopreservation for fish embryos in aquaculture to synchronize the development of embryos derived from different spawning dates or to delay the development for extended periods of time. Maddock [2] demonstrated that in the brown trout (*Salmo trutta*), embryo development can be delayed for up to 4 months at 1.4°C. Studies in the vendace (*Coregonus albula*) showed that hatching of embryos can be delayed for several weeks by cooling them at 1 to 2 °C in order to synchronize mass hatching for lake stocking [23]. In the present study, experiments were carried out in order to optimise the chilled storage protocol for zebrafish embryos

for 18 h at 0°C. Different mixtures of cryoprotectants were used in the chilling medium. Once embryo hatching rates were optimised for 18 h chilling at 0°C, further studies on the effect of 18 h chilling in the presence of cryoprotectant mixture on the gene and protein expressions were carried out. This study was necessary as it has been shown that short term (3 h) chilling of embryos at 0°C does alter the patterns of gene and protein expression [8] . The chilling conditions applied in the present studies have practical applications in fish embryo handling in aquaculture e.g. embryo transportation between fish farms in genetic improvement programs. Storage of embryos at 0°C for prolonged periods of time slow down embryo development significantly therefore providing an alternative method for assessing early stage embryos.

Studies on the effect of 18 h chilling on hatching rate in zebrafish embryos in the presence of sucrose and MeOH mixture

Embryos (50% epiboly) were chilled at 0°C for 18 h with different concentrations of MeOH (0.2, 0.5 and 1 M) in combination of sucrose (0.05 or 0.1M). It was observed that hatching rates increased (56 ±5% and 45 ± 10%) significantly in embryos that had been chilled with 1 M or 0.5 M MeOH in 0.1 M sucrose when compare to those chilled without cryoprotectants. Methanol has been widely used as a cryoprotectant in zebrafish embryo cryopreservation and chilling sensitivity studies [3, 19, 20, 24-27]. In our previous study [28], no significant improvement compared to non-chilled controls (was observed in embryos that were chilled for 18 h in 1 M MeOH) and embryo hatching rate was as low as 5% after chilling at 0°C. It is clear from the present study that 1 M MeOH along with sucrose (0.1 M) supplementation resulted in higher hatching rates than obtained when methanol alone was used during longer term chilling of embryos for up to 18 h at 0°C. Zhang and Rawson [3] obtained an 88.4% hatching rate for heartbeat stage zebrafish embryos that had been chilled at 0°C for 18 h in 1 M MeOH+0.1 M sucrose and the hatching rate decreased to 81% as the chilling period increased to 24 h. In the present study, a 56 ± 5% hatching rate was obtained for 50% epiboly stage embryos under similar conditions. Decreased hatching rates in 50% epiboly embryos compared to the heartbeat stage could be due to the fact that early staged embryos are more sensitive to chilling than late stage

302 embryos and chilling sensitivity decreases with development of embryonic stages[3]. Chilling
303 sensitivity in early stage zebrafish embryos is largely associated with large amounts of intraembryonic
304 lipids [24]. Lipids are the second most abundant component of fish eggs after protein and account for up
305 to 52% of the dry weigh of fish eggs [3]. As the stages progress, yolk is consumed and reduced which
306 makes later staged embryos less prone to chilling. Studies in medaka embryos [6] (12 h hpf – early
307 gastrulation stage which is similar to 50% epiboly in zebrafish) also showed a 3-6 % increase in
308 hatching rates of embryos chilled up to 72 h at 0°C in methanol (3 M) supplemented with sucrose (0.5
309 M) when compared to those obtained from embryos chilled without sucrose. Addition of sucrose in
310 methanol also increased the survival of mrigal (*Cirrhinus mrigala*) embryos from 0% to 25% for tail-
311 bud stage embryos at 4°C for 12 h [29]. This indicated the beneficial effect of sucrose when used in
312 combination with MeOH in protecting embryos from chilling injuries. The protective effect of sucrose
313 may be related to the moderate level of dehydration of embryos in the presence of sucrose which
314 might help in protecting the cell membrane from chilling injury [4]. The mechanisms of sugars such as
315 trehalose (which is similar to sucrose – both are disaccharides) in protecting cells from injuries at low
316 temperatures have been proposed by a number of studies. Trehalose acts as a compatible solute and is
317 an excellent protector of membrane and proteins, it protects membranes during dehydration by
318 hydrogen bonding to the phospholipid head group [30]. This interaction increases head-group spacing,
319 hence lowering the transition temperature of the phospholipids [31]. Unlike methanol, which penetrates
320 into the cell membrane and protects cells intracellularly, sucrose does not penetrate the cell membrane
321 and functions at the extracellular surface of the cells. At this site, the disaccharides increase the
322 osmolarity of medium, thereby preventing fewer water molecules to contact the cell exterior. This
323 process reduces the effect of low temperature on cells and stabilises cell membranes and proteins[32].
324 However, a high concentration of sucrose (1 M) alone caused 100% mortality of early gastrula stage
325 common carp embryos after 12 h storage at 0°C [33]. This may be a result of severe dehydration of the
326 embryos caused by high osmolarity of the sucrose solution.

Studies on the effect of 18 h chilling on *sox* gene and protein expression in zebrafish embryos

Previously, it was observed that 1 M MeOH supplemented with 0.1 M sucrose provided improved hatching rates when embryos were chilled for 18 h at 0°C. The present studies were carried out to investigate whether the use of this cryoprotectant mixture had any effect on *sox* gene and protein expression in embryos that had been chilled for 18 h at 0°C. No significant differences were observed in *sox3* gene expression (Fig 2b) and protein expression (Fig 4) between 18 h non-chilled control embryos and chilled embryos with or without cryoprotectant. However, significant decreases were observed in *sox2* gene (Fig 2a) and protein expression (Fig 4) in 18 h chilled embryos without cryoprotectant when compared to non-chilled 18h control. No significant differences were observed in the 18 h chilled embryos with 1 M MeOH when compared with 18 h RT control. Previous hatching rate results showed improved hatching rate in the embryos that had been chilled for 18 h at 0°C with 1 M MeOH+0.1 M sucrose cryoprotectant mixture compared to other combination of MeOH and sucrose. Chilling for prolonged periods can cause indirect chilling injury [34]. Enzymes are affected by indirect chilling injury at low temperature which may have downstream effects on their function and the metabolic pathways. They contribute to the disorder of metabolic and enzymatic processes can be detrimental in fast developing embryos like *Drosophilla* and such injury increases rapidly at lower temperatures due to loss of co-ordination with reducing temperature[35]. Moreover, reduced temperature may also have adverse effects on the cytoskeleton system i.e. depolymerisation of microtubules [36, 37] which could result in irreversible disruption of cellular process like cell division in oocytes [38, 39]. Damage to the lamina (a part of nuclear envelope) has been reported to affect gene expression as proteins within these also function as structural nuclear protein and are involved in regulation of gene expression [40]. Laminae have also been found to be associated with heterochromatin, at sites of DNA replication, RNA processing, replication protein and RNA polymerases. Any changes in lamina due to chilling injury may have affected such processes and could result in alteration in mRNA transcription and subsequent translation. If such damage occurs, cells would appear morphologically normal following warming, however, future development of the cells could be compromised [40]. Changes in

gene expression also affect the protein expression. In this study, it was observed that decreased *sox2* gene transcripts after 18 h chilling coincided with decreased *sox2* protein expression. This change in protein expression could be mainly due to changes in mRNA transcript, which is needed to initiate protein synthesis. Changes in transcription and translation levels also affect phenotypical changes in embryo development as hatching rates were decreased significantly. Gene and protein expression of *sox2* did not show any decrease in 18 h chilled embryos in 1 M MeOH + 0.1 M sucrose and remained stable compared to 18 h non chilled control. Relatively stable gene and protein expression is reflected in high hatching rates in the embryos.

Studies on the effect of 18 h chilling and warming on *sox* gene and protein expression in zebrafish embryos

In the previous section, it was discussed that 1 M methanol plus 0.1 M sucrose protected gene and protein expression of the embryos following chilling for 18 h. When chilled embryos were subsequently cultured to the hatching stages at 27± 1℃, *sox2* and *sox19a* gene expression was increased significantly at the heartbeat stage and just after 18 h chilling respectively, when compared to non-chilled controls before decreasing to non-chilled control levels by the hatching stage. However, no significant differences were observed at the protein level for *sox2*. No significant differences was observed in *sox3* protein expression in the hatched embryos chilled for 18 h in 1 M MeOH +0.1 M sucrose compare to non-chilled 18 h controls. Results obtained from this study supported by higher hatching rate in the embryo that had been chilled for 18 h in cryoprotectant mixture and cultured to hatching stage, despite the changes in *sox3* gene expression in hatched embryos. Sox genes have capability to bind with other transcription factor proteins to activate or repress specific target genes[41, 42]. A study in early blastula to gastrula stage zebrafish embryos by a quadruple knockdown technique demonstrated that B1 *sox* genes (*sox2/sox3/sox19a/sox19b)* are highly redundant and their encoding proteins are functionally interchangeable in early zebrafish embryogenesis [43] . Therefore changes in *sox3* gene expression could have been compensated by the increased expression of *sox2* and *sox19a* genes observed here. Stable *sox3* protein expression in hatched embryos after they were chilled for 18 h in cryoprotectant mixture when compare to non-chilled control could be due to the post

transcriptional repair mechanism which involves the post transcription and translation modification to repair the loss of *sox3* mRNA transcript due to chilling for 18 h. Similar mechanism explained in parasite *Giarida* after chilling for 20 min on ice demonstrated loss of *hsp90* transcript which was later repaired by the post transcriptional repair mechanism, mRNA *trans*-splicing [44].

To summarise, a 18 h chilled storage protocol for early stage zebrafish embryos was developed that did not significantly compromise *sox* gene expression. 1 M MeOH + 0.1 M sucrose cryoprotectant mixture was found to improve embryo hatching rates following chilling for 18 h at 0°C compared to embryos that had been chilled in 1 M MeOH + 0.05M sucrose or in MeOH alone. This indicated the beneficial effect of sucrose when used in combination with MeOH in preventing embryos from chilling injuries and disruption of the embryonic transcriptional regulators. However, this work was limited to three genes. More work is therefore needed with a larger number of gene sets to have overall picture of embryonic development using this protocol. Another area of study would be to assess changes in gene and/or protein expression in specific areas within embryos to check for any localized changes in transcriptional response.

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Conflict of Interest: None declared.

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501

502

Figure and table legends

Table 1

Information on gene name, accession ID and primer sequences including annealing temperature and product size.

Figure 1: Effect of chilling on hatching rate in 50% epiboly stage of zebrafish embryos

Embryos were chilled for 18 h in different combinations of MeOH and sucrose followed by culturing at 27±1°C for up to three days and until they hatched. Bars represent hatching rates of zebrafish embryos after chilling at 0° C for 18 h in different concentrations of MeOH (0.2, 0.5 and 1 M) plus sucrose (0.05 – 1 M), followed by incubation at 27±1°C for three days. Error bars represent the standard error of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different chilling treatments (n=9).

Figure 2: Effect of chilling in the presence of MeOH and sucrose on *sox* gene expression in 50% epiboly stage of zebrafish embryos

Gene expression profile for *sox2* (a), *sox3* (b) and *sox19a* (c) for embryos that had been chilled for up to 18 h at °C with or without MeOH + sucrose. Non chilled control embryos were kept at 27±1°C. Gene expressions were assessed by reverse transcriptase qPCR. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the non chilled controls and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different conditions of chilling within the same developmental gene (n=9).

Figure 3: Effect of chilling in the presence of MeOH + sucrose and warming on *sox* gene expression in 50% epiboly stages of zebrafish embryos:

Gene expression profile for *sox2* (a), *sox3* (b) and *sox19a* (c) for embryos chilled up to 18 h at °C with or without MeOH + sucrose followed by warming at 27±1°C up to hatching stage, assessed by reverse

transcriptase qPCR. Non chilled control embryos were kept at 27±1°C. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to o h time point and error bars represent standard errors of the mean (SEM). Asterisk (*) shows significant differences ($p < 0.05$) in gene expression between control treatment within the same developmental gene (n=9).

Figure 4: Effect of 18 h chilling with or without 1 M MeOH and 0.1 M sucrose mixture on *sox2* and *sox3* protein expression in 50% epiboly stage zebrafish embryos

Protein expression profiles of *sox2* and *sox3* for embryos chilled for 18 h at 0°C were assessed by western blot. For each time point, 75 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the control at time zero and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different chilling treatments of zebrafish embryos within developmental stages (n=9)

Figure 5: Effect of 18 h chilling with or without 1 M MeOH and 0.1 M sucrose mixture and warming on *sox2* and *sox3* protein expression in hatching stage zebrafish embryos

Protein expression profiles of *sox2* and *sox3* for embryos chilled and warmed until hatching stage for 18 h at 0°C were assessed by western blot. For each time point, 75 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different chilling treatments of zebrafish embryos within developmental stages (n=9)

Table 1 Information on gene name, accession ID and primer sequences including annealing temperature and product size.

Gene Name	Accession ID	Forward/Reverse Primers	Annealing Temp. (°C)	Amplicon size (bp)
sox2	NM_213118.1	F :CTCGGGAAACAACCAGAAAA R: TCGCTCTCGGACAGAAGTTT	58	171
sox3	NM_001001811.2	F: ACCGAGATTAAAAGCCCCAT R: TTGCTGATCTCCGAGTTGTG	56	182
sox19a	NM_130908.1	F: TGTCAACAGCAACAACAGCA R: GTTGTGCATTTTGGGGTTCT	57	126
EF1 - α	NM_131263.1	F: CTGGAGGCCAGCTCAAACAT R: ATCAAGAAGAGTAGTACCGCTAGCATTAC	60	87
β actin	NM_181601.3	F: CGAGCTGTCTTCCCATCCA R: TCACCAACGTAGCTGTCTTTCTG	59	86

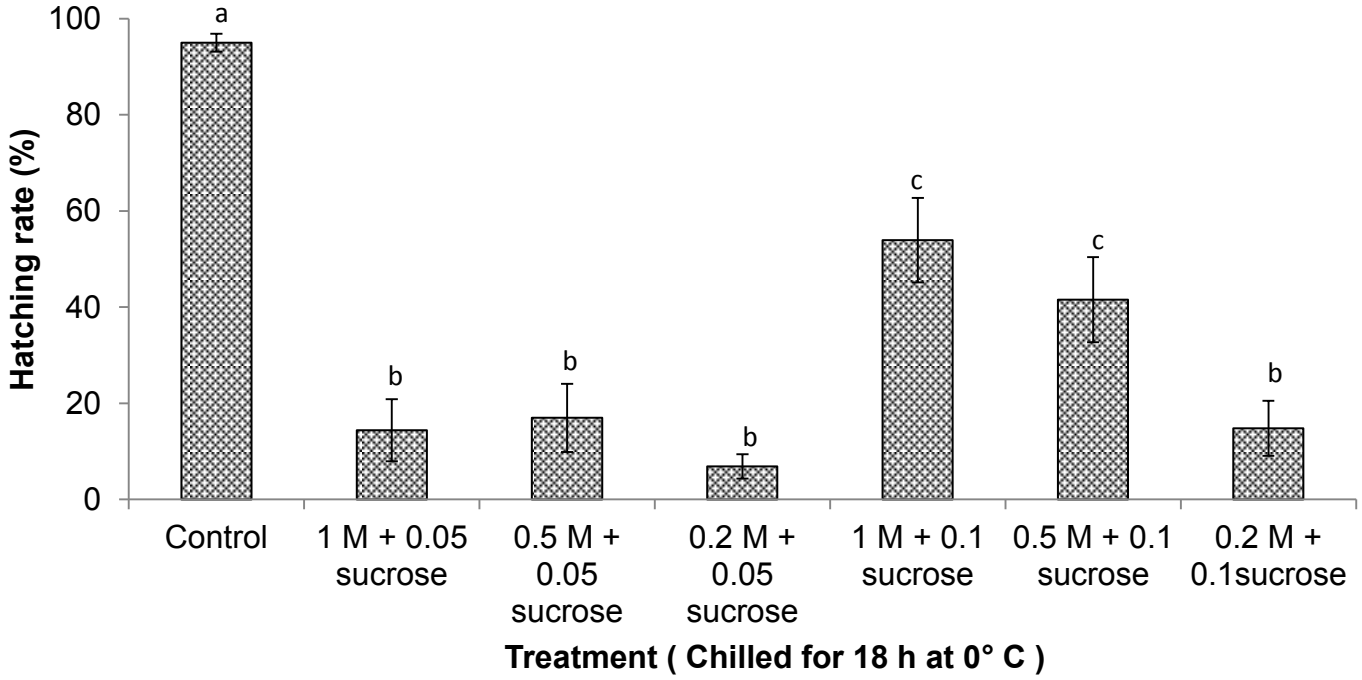
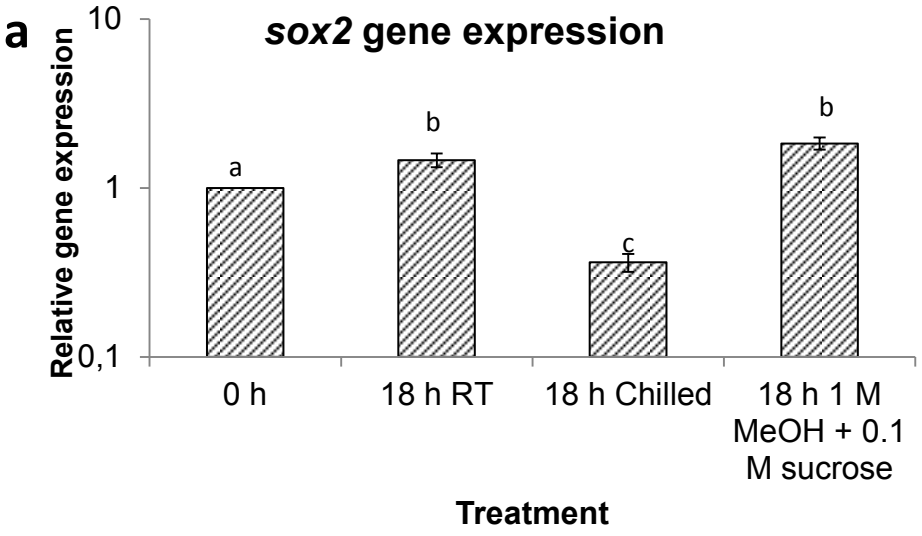
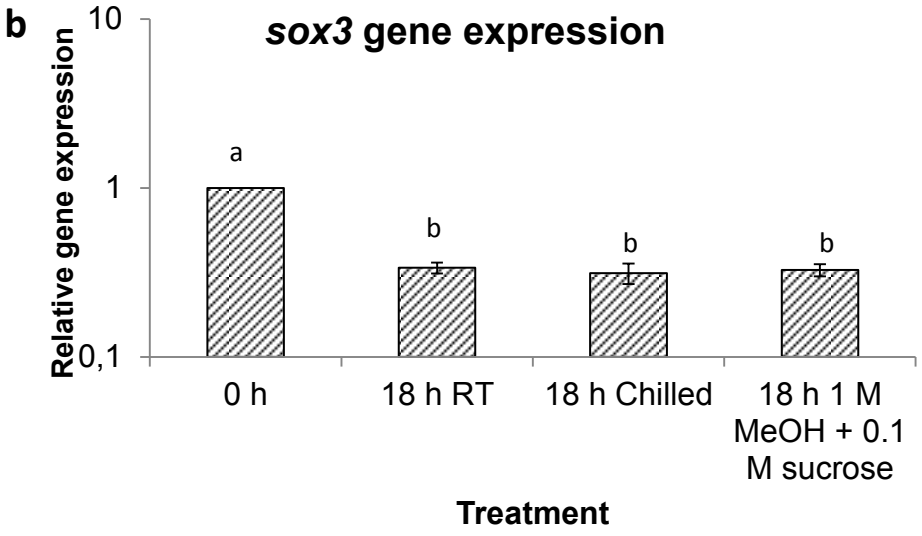

Hatching rate (%)
100
80
60
40
20
0
a
b
b
b
c
c
b
Control
1 M + 0.05 sucrose
0.5 M + 0.05 sucrose
0.2 M + 0.05 sucrose
1 M + 0.1 sucrose
0.5 M + 0.1 sucrose
0.2 M + 0.1sucrose
Treatment (Chilled for 18 h at 0° C)

Figure 1


a
sox2 gene expression
Relative gene expression
10
1
0,1
a
b
c
b
0 h
18 h RT
18 h Chilled
18 h 1 M MeOH + 0.1 M sucrose
Treatment


b
sox3 gene expression
Relative gene expression
10
1
0,1
a
b
b
b
0 h
18 h RT
18 h Chilled
18 h 1 M MeOH + 0.1 M sucrose
Treatment

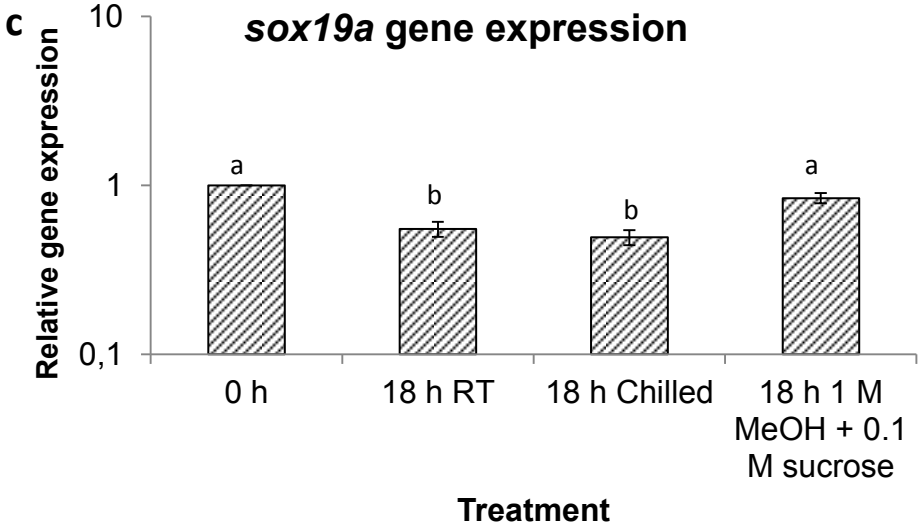
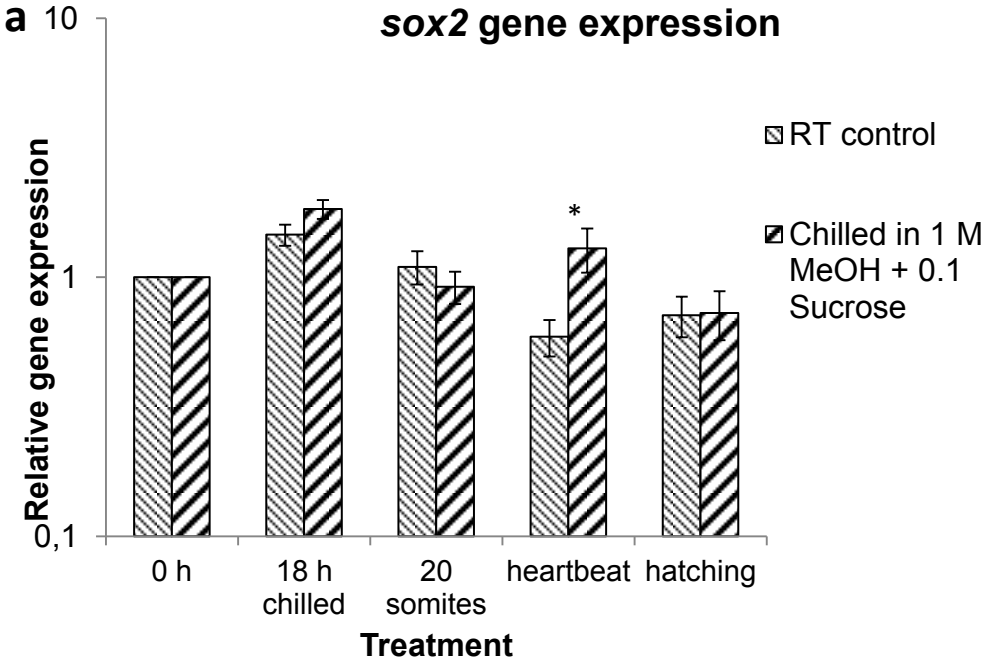
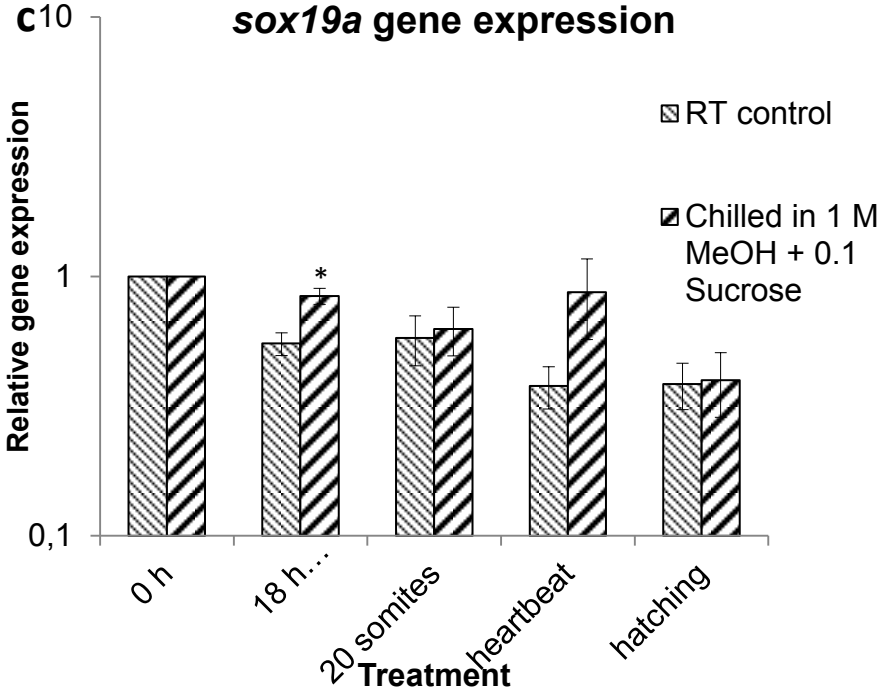

c
sox19a gene expression
Relative gene expression
10
1
0,1
a
b
b
a
0 h
18 h RT
18 h Chilled
18 h 1 M MeOH + 0.1 M sucrose
Treatment

Figure 2

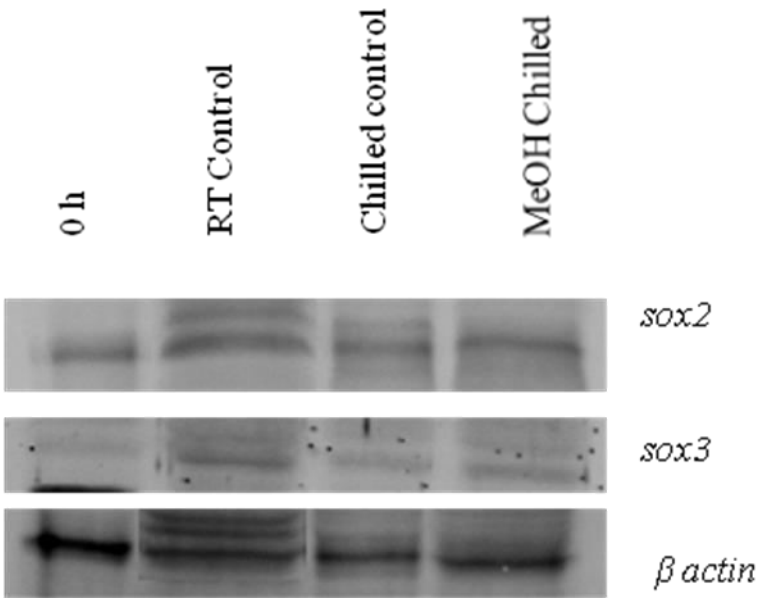

a
sox2 gene expression
10
1
0,1
Relative gene expression
RT control
Chilled in 1 M MeOH + 0.1 Sucrose
*
0 h
18 h chilled
20 somites
heartbeat
hatching
Treatment


c
sox19a gene expression
10
1
0,1
Relative gene expression
RT control
Chilled in 1 M MeOH + 0.1 Sucrose
*
0 h
18 h…
20 somites
heartbeat
hatching
Treatment

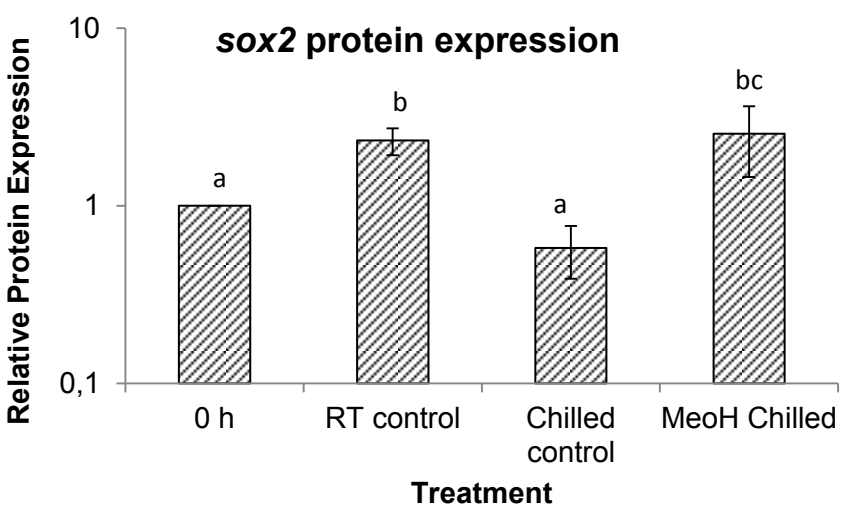

b
sox3 gene expression
10
1
0,1
Relative gene expression
RT control
Chilled in 1 M MeOH + 0.1 M Sucrose
*
*
0 h
18 h chilled
20 somites
heartbeat
hatching
Treatment

Figure 3

(a)


0 h
RT Control
Chilled control
MeOH Chilled
sox2
sox3
β actin

(b)


sox2 protein expression
Relative Protein Expression
10
1
0,1
a
b
a
bc
0 h
RT control
Chilled control
MeoH Chilled
Treatment


sox3 protein expression
Relative Protein Expression
10
1
0,1
a
bc
ac
bc
0 h
RT control
Chilled control
MeoH Chilled
Treatment

Figure 4

(a)

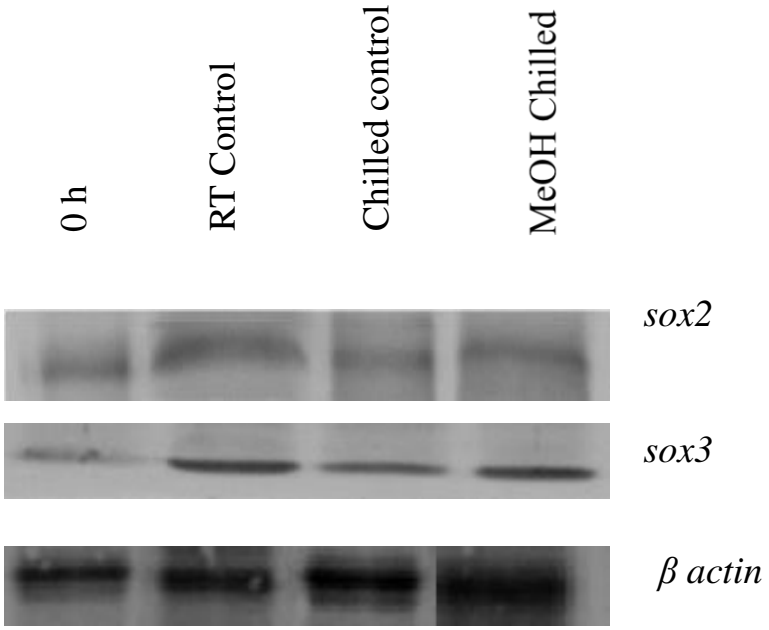
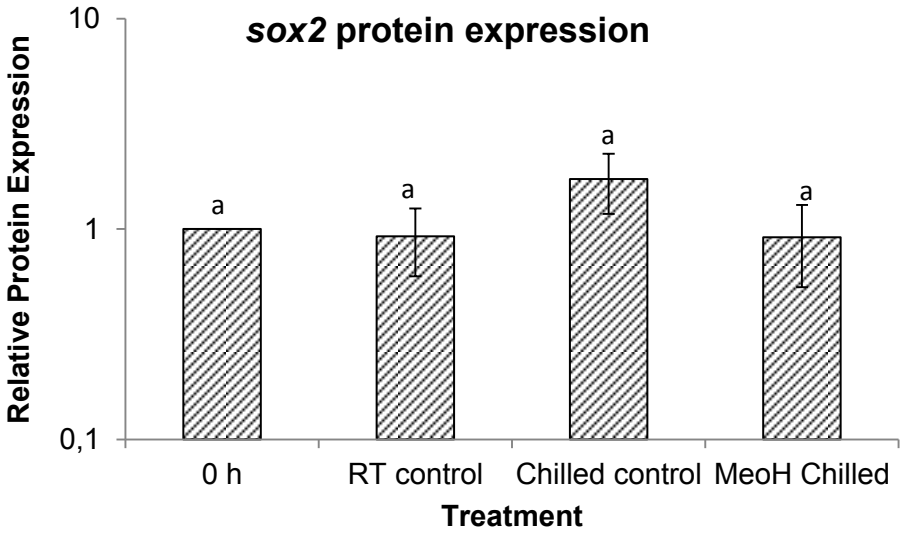

0 h
RT Control
Chilled control
MeOH Chilled
sox2
sox3
β actin

Figure 5

(b)


sox2 protein expression
Relative Protein Expression
10
1
0,1
a
a
a
a
0 h
RT control
Chilled control
MeoH Chilled
Treatment


sox3 protein expression
Relative Protein Expression
10
1
0,1
a
b
b
b
0 h
RT control
Chilled control
MeoH Chilled
Treatment