Cryobiology 71 (2015) 1–11

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Use of methanol as cryoprotectant and its effect on *sox* genes and proteins in chilled zebrafish embryos ☆

Kunjan Desai [a], Emma Spikings [b], Tiantian Zhang [c,*]

[a] Department of Neuroscience and Regenerative Medicine, Georgia Regents University, Augusta, GA 30912, USA
[b] Institute of Biomedical Science and Environmental Science and Technology, University of Bedfordshire, Luton, Bedfordshire LU2 8DL, UK
[c] School of Applied Science, Bournemouth University, Poole BH12 5BB, UK

ARTICLE INFO

Article history:
Received 29 April 2015
Revised 23 June 2015
Accepted 24 June 2015
Available online 26 June 2015

Keywords:
Chilling
Zebrafish
50% epiboly embryo
Methanol effect
Hatching
Sox gene expression
Protein expression

ABSTRACT

Methanol is a widely used cryoprotectant (CPA) in cryopreservation of fish embryos, however little is known about its effect at the molecular level. This study investigated the effect of methanol on sox gene and protein expression in zebrafish embryos (50% epiboly) when they were chilled for 3 h and subsequently warmed and cultured to the hatching stages. Initial experiments were carried out to evaluate the chilling tolerance of 50% epiboly embryos which showed no significant differences in hatching rates for up to 6 h chilling in methanol (0.2-, 0.5- and 1 M). Subsequent experiments in embryos that had been chilled for 3 h in 1 M methanol and warmed and cultured up to the hatching stages found that *sox2* and *sox3* gene expression were increased significantly in hatched embryos that had been chilled compared to non-chilled controls. *Sox19a* gene expression also remained above control levels in the chilled embryos at all developmental stages tested. Whilst stable sox2 protein expression was observed between non-chilled controls and embryos chilled for 3 h with or without MeOH, a surge in sox19a protein expression was observed in embryos chilled for 3 h in the presence of 1 M MeOH compared to non-chilled controls and then returned to control levels by the hatching stage. The protective effect of MeOH was increased with increasing concentrations. Effect of methanol at molecular level during chilling was reported here first time which could add new parameter in selection of cryoprotectant while designing cryopreservation protocol.

1. Introduction

Methanol has been a widely used cryoprotectant in the cryopreservation of embryos and oocytes and other reproductive tissues. However, success of most of cryopreservation protocols is usually measured by either physical appearance of cell or survival rate. It has previously been reported that chilling alters the pattern of *sox* gene expression in zebrafish embryos [8]. Similarly, the effect of cryoprotectant use at the molecular level is still unknown. In the present study, the effect of chilling in the presence of cryoprotectant methanol on gene and subsequent protein expression was investigated.

Cryoprotectants usually protect cells from chilling and freezing injury by dehydrating cells and lowering the freezing point [35]. The use of cryoprotectants in low temperature storage has been proven to be essential in protecting cells from chilling injury [49]. However, most cryoprotectants are toxic especially when used at high concentrations [45]. The toxicity of cryoprotectants to cells is also dependent on their type, exposure temperature and exposure time period [40]. Cryoprotectants can cause cellular injury by osmotic trauma [34] and can be toxic to the cells. Cryoprotectant toxicity studies are now common practice prior to their use in cell cryopreservation. However there is very limited information on how cryoprotectants function at the molecular level and if they have a significant effect on gene or protein expression following cryopreservation. Understanding of the impact of cryoprotectants at the molecular level is important especially for reproductive materials such as embryos, oocytes, ovarian tissues. Any changes at the molecular level could have a lethal effect on subsequent development. Any alteration during these early stages could be replicated leading to long term genetic defects. Studies in mouse and rat embryos have shown that methanol (MeOH) is toxic

☆ *Statement of funding:* Funding support for this programme of research was provided by the strategic research fund of the Institute of Biomedical and Environmental Science and Technology, University of Bedfordshire, Luton, UK.

* Corresponding author.
E-mail addresses: kunjandesai@live.com (K. Desai), emma.spikings@beds.ac.uk (E. Spikings), tzhang@bournemouth.ac.uk (T. Zhang).

http://dx.doi.org/10.1016/j.cryobiol.2015.06.009
0011-2240/

[22] and even lethal when used at high concentrations (12–16 mg/mL) [2]. Methanol is a widely used cryoprotectant in fish embryo cryopreservation. Methanol has been found to protect cells during cryopreservation in zebrafish oocytes and embryos [43,49] and common carp embryos [1]. It has been found that methanol was effective in zebrafish embryo cryopreservation because it has low toxicity compare to other most commonly used cryoprotectants [49] and also due to its ability to pass through the embryo membrane rapidly [17]. Similar studies in medaka also demonstrated higher embryo survival rates after chilling in the presence of MeOH [45] than chilling in ethylene glycol (EG), propylene glycol (PG), DMSO and glycerol. However, it has also been shown that methanol exposure is associated with visual impairment or blindness, affecting the optic nerve and retina of rats at concentrations 20% w/v of 4 g/kg followed by 2 g/kg [12]. Methanol has also been demonstrated to be neurotoxic where its exposure leads to severe central nervous system (CNS) defects in mice at gastrulation periods [7] and in drosophila embryos at 8–11 embryonic stages [26]. Rico *et al.* showed that methanol also alters ecto-nucleotidases and acetylcholinesterase enzymes (important for neuromodulation in brain) in zebrafish brains [36]. Therefore it is important that the effect of methanol is better understood when used as a cryoprotectant.

The present study investigated the effect of chilling on *sox* gene and protein expression in the presence of methanol. *Sox* genes (*sox2*, *sox3* and *sox19a*) are important genes in the development of nervous systems in zebrafish embryos and any changes can lead to serious abnormalities [13]. Inhibition of *sox* gene expression in vertebrate embryos results in premature differentiation of neural precursors and their overexpression results in inhibition of neurogenesis [4,5,15,19,32]. However, study on gene expression (mRNA level) does not provide information on protein translation as the efficacy of translation can also be affected by post transcription modulation of regulatory genes [25]. It has been demonstrated that small non-protein-coding RNAs (small nucleolar RNA, micro RNAs, short interfering RNAs, small double stranded RNA) also regulate gene expression, including translation in developmental processes [25]. Therefore, following gene expression studies, subsequent protein expression studies were also carried out to understand the effect of MeOH at the molecular level during cryoprotectant exposure and chilling. For these study, embryos were chilled for up to 24 h to find out optimum chilling storage period (commonly used transportation period) using MeOH as cryoprotectant. It is also further necessary to test optimum concentration which not only improve survival rate but also does not change anything at genetic level, especially neurological genes and proteins i.e. *sox*, due to their neurotoxic tendency. Once this condition has been optimised, further cryopreservation protocol would be developed by addition of sugars without compromising genetic integrity.

2. Materials and methods

2.1. Zebrafish maintenance and embryo selection

All the procedures and protocol carried out in this study were ethically approved by LIRANS institute of Research in the Applied Natural Science's (LIRANS) Ethical Board Committee and University of Bedfordshire Ethical Scrutiny Committee. Adult zebrafish 12–14 weeks old were maintained in 40 L glass tanks at 27 ± 1 °C. The males and females were kept at a ratio of 1:2 and a 12 h light/dark cycle was used. Fish were fed 3 times a day with TetraMin® (Tetra, Germany) and once a day with freshly hatched brine shrimp (*Artemia salini*) (ZM systems, UK). Embryos were collected in the morning and kept in a 27 ± 1 °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].

3. Experimental design

3.1. Experiment 1: Impact of chilling at 0 °C on embryo hatching rate for different time periods in the presence of MeOH as a cryoprotectant

Embryos (50% epiboly) were chilled [23] at 0 °C in crushed ice (temperature was maintained throughout by addition of ice) for up to 24 h (3-, 6-, 18- and 24-h) in the presence of different concentrations of MeOH (0.2, 0.5 and 1 M). After chilling, cryoprotectant MeOH was replaced with egg water (60 μg/mL sea salt in distilled water) and the test tubes were quickly placed into a 27 ± 1 °C water bath and embryos were then incubated at 27 ± 1 °C for up to 3 days or until they hatched. Control embryos were kept at 27 ± 1 °C and incubated for 3 days or until they hatched. Hatching rates were then monitored and all experiments were repeated 3 times in triplicate (total embryo = 2025). Embryos were considered to be hatched when their chorion is missing, there were no obvious signs of malformation, and there was natural movement with a functional heartbeat. Embryos were considered unhatched if they showed no signs of cell differentiation, yolk coagulation and no tail formation or detached tail and/or if they remained in the chorion [21].

3.2. Experiment 2: Impact of chilling and warming on sox gene expression in zebrafish (Danio rerio) embryos in the presence of MeOH

Based on the results obtained from the previous experiments, further studies were carried out on the effect of 3 h chilling on gene and protein expression in 50% epiboly embryos in the presence of MeOH.

3.2.1. Chilling of embryos

Embryos at 50% epiboly stage were chilled at 0 °C for 3 h with different concentrations of MeOH (0.2, 0.5 and 1 M) as described above. RNA was then extracted and cDNA was produced as described below [8]. cDNA was diluted 1:2 with molecular biology grade water (Sigma, UK) for use in real time PCR. For each time point, three different biological samples (5 embryos/tube) were treated and stored. Each experiment was repeated 3 times. Experimental controls were kept at 27 ± 1 °C in a water bath for the equivalent time period.

3.2.2. Warming and incubation of embryos after chilling

Embryos at 50% epiboly stages were chilled for 3 h at 0 °C, they were then warmed up and incubated at 27 ± 1 °C until three key developmental stages –20 somites stage (hind brain development), heartbeat stage (first heart beat starts) and hatching stages (first time when actual larvae exposed to environment). For each embryonic stage, three different samples (5 embryos/tube) were treated and stored for RNA extraction at –80 °C and real time PCR. Each experiment was repeated 3 times. Experimental controls were kept at 27 ± 1 °C in water bath.

3.3. Experiment 3: Impact of MeOH chilling and warming on sox2 and sox19a protein expression in zebrafish (D. rerio) embryos

Embryos (75 embryos) from 50% epiboly stages were chilled for 3 h with/without 1 M MeOH and then returned to 27 ± 1 °C and incubated until they hatched. Cryoprotectant solution was replaced by 27 ± 1 °C egg water (60 μg/mL sea salt in distilled water) following chilling before incubation. Experimental controls