

Manipulated Mouse Embryos as Bioassay System for Water Quality Control

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Contents

Mouse pronuclear stage embryos with intact slit zona pellucida (manipulated) were cultured *in vitro* until the hatched blastocyst stage in simplex optimized medium with higher K⁺ concentration (KSOM) prepared with three different water types: tap, deionized reverse osmosis (D/O) water and Milli-Q system (M/Q) water. The culture media were supplemented with or without protein and ethylenediaminetetraacetic acid (EDTA, disodium salt). The rates of hatched blastocysts were significantly affected ($p < 0.01$) by micromanipulation, protein supplement and water source. The water source has no influence ($p > 0.05$) on development in EDTA supplemented protein free culture media, whereas in EDTA and protein free culture media, the water quality significantly ($p < 0.001$) affected the rates of development, with higher rates in media prepared with M/Q water. The micromanipulated embryos showed higher sensitivity to the water quality ($p < 0.01$). It worth mentioning that the rates of hatched blastocysts in protein free culture media were very low (0–7.5%). Furthermore, the three different water types were analysed by measuring the electrical conductivity, inorganic ions, total organic carbon and endotoxins to evaluate the purity. M/Q water showed the lowest levels of inorganic ion, total organic carbon and endotoxin concentrations. We concluded that manipulated mouse embryos are good system to evaluate the quality of water used in biological system.

Introduction

The quality of water used in culture media preparation differs, in different laboratories, according to the purification method and the equipment. Several studies have examined the effects of water quality on the development of mouse embryos (Whittingham 1971; Fukuda et al. 1987; George et al. 1989; Flood and Shirley 1991; Fleetham et al. 1993) and bovine embryos (Nagao et al. 1995). These studies showed that the development of embryos was significantly affected by the quality of water used to prepare culture media. However, the culture media used in these studies contained chelating agents such, as proteins (Fukuda et al. 1987; George et al. 1989) and amino acids (Nagao et al. 1995). These chelating substances abolish the effects of embryotoxic substances in culture media (Lindenbaum 1973; Flood and Shirley 1991). Ethylenediaminetetraacetic acid (EDTA), which is widely used in murine culture media, is also a strong chelator that modulates the effects of toxic substances in water (Nasr-Esfahani et al. 1990). Furthermore, endotoxins in culture media have been reported to affect the development of human embryos (Fishel et al. 1988) and to cause atresia of bovine follicles cultured in the absence of proteins (Bosu et al. 1996; Perez et al. 1996).

To increase the sensitivity of the quality control of culture media, some researchers have proposed using

mouse zona pellucida-free embryos as test material (Fleming et al. 1987; Fleetham et al. 1993). However removing zona pellucida affects the cell contact at the 4-cell stage (Elsheikh et al. 1997) and the inner cell mass differentiation, which leads to poor development *in vitro* (Suzuki et al. 1995).

This study was carried out to find a suitable bioassay system for water quality control. Three different types of water were used to prepare culture media. The culture media were supplemented with protein and/or EDTA. Murine pronuclear stage embryos with intact and slit zona pellucida were cultured in these culture media and their development to the hatched blastocyst stage was assessed. Furthermore, the three different water types were analysed to study the mutual relation between water quality and the method of purification.

Materials and Methods

Preparation of culture media

KSOM supplemented with 0.01 mM EDTA (Erbach et al. 1994) was prepared weekly for each trial with three different types of water: tap water from our laboratory, deionized reverse osmosis (D/O) water, and Milli-Q water (M-Q), which is tap water purified by the Millipore water purification system (Milli-Rx12 plus and Milli-Q SPTOC with an ultra-filter; Nihon Millipore Ltd, Tokyo, Japan). The media were supplemented with 1 mg/ml polyvinyl alcohol (PVA; Sigma Chemical Co., St Louis, MO, USA) when used (Nagao et al. 1995), except in experiment 1, in which media were supplemented with either PVA or 1 mg/ml bovine serum albumin (BSA, A7030; Sigma). All media were filtered through a filter sterilizer (Gelman Sciences, Ann Arbor, MI, USA), and 30 µl drops were placed on sterilized tissue culture dishes (35 × 10 mm; Nunc, Roskilde, Denmark) and were covered with paraffin oil. For embryo collection, from KSOM prepared with M-Q water and supplemented with 1 mg/BSA, 3 ml were placed in tissue culture dishes (35 × 10 mm; Nunc) and were covered with oil. The media were kept over night in a CO₂ incubator at 37°C under 5% CO₂ in air until use.

Water analysis

The electrical conductivity values of the three water types were measured by a portable conductivity meter (CM-1K; Toa Co. Ltd., Tokyo, Japan). An organic carbon analyser (Toc-5000 A; Shimadzu Corp., Tokyo, Japan) was employed to analyse the total organic carbon of each sample. An ion chromatographic analyser (IC 7000 Series II; Yokogawa Co., Tokyo, Japan)

was used to analyse the concentrations of sodium, potassium, calcium and magnesium. Chloride ions were measured with an ion chromatographic analyser (IC 7000 Series, Yokogawa), and the concentrations of iron and zinc ions were measured by an atomic absorption spectrophotometer (180-80 S; Hitachi Ltd, Tokyo, Japan). The endotoxin concentration was measured by a Toxicator endotoxin kit (Toxicolor system LS-6 Set, Seikagaku Corp., Tokyo, Japan).

Embryo collection

At 6–8 weeks of age, F1 female mice (C57 BL X CBA) were superovulated with intraperitoneal injections of 5 IU of pregnant mares' serum gonadotropin (Serotropin; Teikoku Hormone Hfg. Co. Ltd., Tokyo, Japan), followed by 5 IU of human chorionic gonadotropin (hCG, Gonatropin; Teikoku Hormone) 48 h later. Immediately after the hCG injection, the females were placed individually in cages of males with proven fertility from the same strain. Fifteen hours after hCG injection they were checked for the presence of vaginal plugs. Females with vaginal plugs were killed by cervical dislocation 22–24 h after hCG. The mice were dissected and the oviducts were placed in KSOM supplemented with 1 mg/ml BSA under paraffin oil. Pronuclear-stage embryos with clear second polar bodies and two pronuclei were collected by flushing the oviducts with KSOM. The cumulus cells were removed from the embryos by repeated pipetting. The pronuclear-stage embryos were thoroughly washed, pooled and kept at 37°C in an atmosphere of 5% CO₂ in air until distributed to the different treatments.

Embryo micromanipulation

From the pooled embryos, the zona pellucidae of some embryos were slit in Dulbecco's phosphate-buffered saline (PBS) supplemented with 4 mg/ml BSA as described by Tsunoda et al. (1986). Briefly, 10–20% of the circumference of the zona pellucidae was slit with a fine glass needle under an inverted microscope (Diaphot; Nikon, Tokyo, Japan) with Nomarski's contrast system and Narishige micromanipulators. The zona pellucida-slit embryos were thoroughly washed and then were cultured in protein-free media as designed (see experiment 3).

Embryo culture

The embryos in the different experiments were cultured for 120 h in 30 µl drops of the different culture media prepared at 37°C under 5% CO₂ in air. The progress of the development towards the hatched blastocyst stage was recorded every 24 h under an inverted microscope at 20–40× magnifications.

Experimental design

Experiment 1

This experiment examined the effects of water type and protein supplementation to the culture media on the development of zona pellucida-intact mouse pronuclear-stage embryos to hatched blastocysts. Embryos from the

pooled pronuclear stage embryos were cultured in EDTA-free KSOM medium prepared with the three water types and were supplemented with 1 mg/ml BSA or PVA as mentioned above, and their developmental progress towards the hatched blastocysts stage was recorded.

Experiment 2

This experiment tested the effects of the presence or absence of EDTA in KSOM prepared with the three different water types on the development of zona pellucida-intact pronuclear-stage embryos to hatched blastocysts. The pronuclear stage embryos were cultured in protein-free KSOM with or without EDTA (0.01 mM) and their development to the hatched blastocyst was recorded.

Experiment 3

This experiment evaluated the effects of water type used for culture media preparation on development of micro-manipulated mouse pronuclear stage embryos to hatched blastocysts. Mouse pronuclear-stage embryos with slit zona pellucidae were cultured in EDTA and protein-free KSOM prepared with the three different water types and were supplemented with 1 mg/ml PVA. Their development to hatched blastocysts was recorded. Zona-intact pronuclear-stage embryos were employed as control.

Statistical analysis

The results were statistically evaluated with two-way ANOVA followed by Fisher's protected least significance difference. Differences at a probability of $p < 0.05$ were considered to be statistically significant.

Results

Experiment 1

Two-way ANOVA showed no interaction between the effects of water type and BSA ($p > 0.05$) on the developmental rates to the 2-cell, morula and blastocyst stages. Neither type of water nor BSA supplementation affected ($p > 0.05$) the development of mouse pronuclear-stage embryos to the expanded blastocyst stage (Table 1). However, water type and BSA supplementation affected the rates of hatched blastocysts ($p < 0.05$).

Experiment 2

Two-way ANOVA showed a significant interaction ($p < 0.05$) between the effects of water type and EDTA supplementation to the culture media. In the presence of EDTA, the water type did not affect the embryonic development ($p > 0.05$), whereas in its absence, the water type affected the development significantly ($p < 0.001$) (Table 2).

Experiment 3

Two-way ANOVA showed a significant interaction ($p < 0.05$) between the effects of water type and zona

Water source ^a	Protein	No. of embryos cultured	% of embryos developed to ^b			
			2-cell (24 h)	Morulae (72 h)	Blastocysts (96 h)	Hatched blastocysts (120 h)
M-Q	+	132	95 ± 3	96 ± 4	91 ± 7	37 ± 4 ^c
		123	97 ± 2	95 ± 2	93 ± 2	7 ± 5 ^d
D-O	+	144	96 ± 2	94 ± 4	93 ± 3	25 ± 1 ^{c,e}
		128	97 ± 1	96 ± 1	91 ± 1	7 ± 4 ^d
Tap	+	138	93 ± 3	92 ± 2	87 ± 2	23 ± 2 ^c
		129	97 ± 1	96 ± 2	90 ± 2	0 ^d

^aM-Q: tap water purified by Millipore system; D-O: deionized reverse osmosis water; Tap: tap water from our laboratory.

^bPercentages were based on the number of the cultured embryos and summarized as mean ± SEM of four replicates. Media were ethylenediaminetetraacetic acid-free.

^cValues with different superscripts in the same column differ significantly (p < 0.05).

Table 1. Effects of water source and protein supplementation to culture media on development of mouse pronuclear stage embryos

Water source ^a	EDTA	No. of embryos cultured	% of embryos developed to ^b			
			2-cell (24 h)	Morulae (72 h)	Blastocysts (96 h)	Hatched blastocysts (120 h)
M-Q	+	89	98 ± 1 ^c	97 ± 2 ^c	93 ± 1 ^c	1 ± 1
		87	99 ± 1 ^c	66 ± 7 ^d	39 ± 3 ^d	0
D-O	+	83	95 ± 3 ^c	95 ± 3 ^c	95 ± 3 ^c	2 ± 1
		87	100 ± 0 ^c	51 ± 10 ^e	18 ± 4 ^e	0
Tap	+	80	97 ± 1 ^c	97 ± 2 ^c	97 ± 2 ^c	1 ± 1
		87	12 ± 1 ^d	0 ^f	0 ^f	0

^aM-Q: tap water purified by Millipore system; D-O: deionized reverse osmosis water; Tap: tap water from our laboratory.

^bPercentages were based on the number of the cultured embryos and summarized as mean ± SEM of four replicates. Media were protein-free.

^cValues with different superscripts in the same column differ significantly (p < 0.05).

Table 2. Effects of water source and ethylenediaminetetraacetic acid supplementation to culture media on development of mouse pronuclear stage embryos

Table 3. Effect of water source and slitting of zona pellucida on development of mouse pronuclear stage embryos

Water source ^a	Zona pellucida	No. of embryos cultured	% of embryos developed to ^b			
			2-cell (24 h)	Morulae (72 h)	Blastocysts (96 h)	Hatched blastocysts (120 h)
M-Q	Intact	62	97 ± 2	97 ± 2 ^c	97 ± 2 ^c	0 ^c
	Slit	38	100 ± 0	100 ± 0 ^c	100 ± 0 ^c	70 ± 15 ^d
D-O	Intact	55	100 ± 0	98 ± 2 ^c	98 ± 2 ^c	0 ^c
	Slit	39	100 ± 0	100 ± 0 ^c	100 ± 0 ^c	33 ± 16 ^e
Tap	Intact	55	98 ± 2	92 ± 3 ^c	91 ± 2 ^c	0 ^c
	Slit	40	95 ± 4	75 ± 7 ^d	66 ± 4 ^d	0 ^c

^aM-Q: tap water purified by Millipore system; D-O: deionized reverse osmosis water; Tap: tap water from our laboratory.

^bPercentages were based on the number of the cultured embryos and summarized as mean ± SEM of four replicates. Media were ethylenediaminetetraacetic acid and protein-free.

^cValues with different superscripts in the same column differ significantly (p < 0.05).

Table 4. Electrical conductivity values and ions, total organic carbon and endotoxin concentrations for the three different types of water

Water type ^a	Electrical conductivity value (μs/cm) ^b	Ion concentration (mg/l) ^c							Total organic carbon (mg/l) ^d	Endotoxin concentration (EU/ml) ^e
		Na	K	Cl	Ca	Mg	Fe	Zn		
Tap	369.9 ± 6.8	19.48	5.14	31.0	28.31	19.53	<0.01	<0.02	0.782	> 2.439
D-O	<4.0	<0.1	<0.1	0.5	<0.1	<0.01	<0.004	0.004	0.429	0.225
M-Q	<4.0	0.02	<0.01	0.5	<0.01	<0.01	<0.004	0.004	0.371	<0.000

^aTap: tap water from our laboratory; D-O water: deionized reverse osmosis water; M-Q water: tap water passed through Millipore system.

^bElectrical conductivity values were mean ± SEM of three trials (μs, microsiemens).

^cIons were measured by ion chromatographic analysers, except iron and zinc which we analysed by atomic absorption.

^dTotal organic carbon was measured by an organic carbon analyser.

^eEndotoxin concentration was measured by a Toxicolor Endotoxin kit (EU, Endotoxin unit).

slitting. The water source did not affect the development of zona pellucida-intact embryos ($p > 0.05$), whereas the development of embryos with slit zona pellucidae was significantly affected ($p < 0.05$) by the type of water (Table 3).

Results of water analysis

The analysis of the three different water types showed that tap water had the highest conductivity; inorganic ions, total organic carbon and endotoxin concentrations (Table 4), whereas M-Q water had the lowest concentrations of inorganic ions, total organic carbon and endotoxin concentrations. This analysis showed that M-Q water had the highest purity among the three different water types.

Discussion

The results of this study showed that the water quality substantially affected the development of murine pronuclear stage embryos with intact or slit zona pellucidae. Protein supplementation to the culture media prepared from different water sources improved the rates of hatched blastocysts. The results also showed that EDTA supplementation modified the effects of water types on development. Furthermore, slitting of zona pellucidae increased the sensitivity of pronuclear-stage embryos to toxic substances in culture media prepared with poor water quality.

The quality of water used to prepare culture media influences *in vitro* development of bovine embryos produced by *in vitro* fertilization in protein-free media (Nagao et al. 1995) and the development of murine 1-cell stage embryos to the blastocyst stage in culture media supplemented with protein (Fukuda et al. 1987); our findings confirmed these results. The improved rates of hatched blastocysts as a result of protein supplementation in our experiment agreed with the results of Wright et al. (1978) who showed that proteins increase the rate of hatched blastocysts of murine embryos. Proteins abolish the effects of toxic substances in culture media (Saito et al. 1984; Naz et al. 1986; Ogawa and Marrs 1987; Flood and Shirley 1991).

The tap water used to prepare the culture media in this study had a high level of endotoxins (> 2.439 EU/ml) and resulted in a significantly lower proportion of hatched blastocysts compared with M-Q water (Table 1). The endotoxins found in the tap water did not seem to cross the zona pellucidae and the embryos were able to develop to the expanded blastocyst stage regardless of the protein supplementation. The low rate of hatched blastocysts in tap water was probably due to the high levels of endotoxins, that entered the embryos immediately before hatching as a result of thinning or perforation of the zona pellucidae and affected the embryo viability.

The water quality in culture media supplemented with EDTA had no effect on murine embryos, whereas in EDTA-free culture media, the water quality considerably affected the development. EDTA is a strong chelator that abolishes the toxic effects of metal ions

in culture media (Lindenbaum 1973; Nasr-Esfahani et al. 1990). As in experiment 2 we excluded BSA from the culture media, the endotoxins and toxic ions may cooperatively suppress the development of mouse embryos. In contrast, George et al. (1989) reported that water quality has no effect on development of mouse embryos cultured in culture media supplemented with BSA. The discrepancy between the two studies may be due to the different water types used, as well as to the BSA and/or EDTA supplementation to the culture media. The results also showed that highly purified water supports the development to the blastocyst stage in EDTA- and protein-free media. The results of our experiment suggest that EDTA supplementation to the culture media modulates the effects of water quality on *in vitro* development.

Mouse embryos with intact zona pellucidae are more resistant to suboptimal culture conditions (Fleming et al. 1987). Removal of zona pellucidae from murine pronuclear-stage embryos increased their sensitivity to poor-quality water used to prepare the culture media (Fleetham et al. 1993). Slitting the zona pellucidae in our study increased the embryo sensitivity to water quality, with a considerable improvement in the rates of hatched blastocysts when M-Q water was used. The reduced rates of hatched blastocysts when tap and D-O water were used were probably due to the high levels of endotoxins, organic compounds and/or inorganic ions that entered the embryos through the slit in the zona pellucidae. This result suggests that micromanipulated embryos are more sensitive to culture media prepared with water containing high levels of toxic substances.

The M-Q system employed in this study is provided with ultraviolet, reverse osmosis and distillation units. These units reduce the bacterial endotoxins, organic compounds, electrical conductivity and inorganic ions. High electrical conductivity, high inorganic ion concentrations and high levels of organic compounds in the water used for culture media preparation reduce embryonic development in cattle (Nagao et al. 1995) and mice (Fukuda et al. 1987). Furthermore, highly purified water was reported to have low levels of endotoxins (Nagao et al. 1995). Our results agreed with these studies (Fukuda et al. 1987; Nagao et al. 1995), but the effects of the water types having these properties were more pronounced when EDTA was excluded from the culture media and the zonae of embryos were slit. The results of our study confirmed that high purification of water is very important to produce water of high quality, free not only from inorganic ions, but also free from organic compounds and endotoxins to allow high rates of *in vitro* development of murine embryos. The different laboratories purify water with different equipment, but in this study we found that the purification system having an ultraviolet unit is more efficient in water purification.

We concluded that micromanipulated mouse embryos are good bioassay system for evaluation of the quality of water used in biological systems. Also the results have shown that the supplementation of chelating substances to culture media modulates the effects of toxic materials in water.

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