Nutritional requirement for elemental iron during preimplantation embryo development in vitro

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Abstract
Objective
To determine (i) the effect of iron depletion on embryo development in vitro; (ii) whether the amount of iron present in embryo culture media is sufficient for embryo development; (iii) the toxic, tolerance, optimal levels of iron on embryo development; and (iv) to examine the impact of supplemented iron on embryo development in vitro.
Methods
Quakenbush mice zygotes were randomly apportioned to individual treatments. Treatments consisted of culture media supplemented with (i) desferrioxamine (DES), (ii) graded amounts of ferric iron without EDTA or (iii) with 0.11mM EDTA. The end point was development in vitro of expanded, hatching, hatched embryos.
Results
DES >1000nM (but not 200-800nM) and ferric iron without EDTA >8µM was toxic. Iron supplementation 2 to 10µM and %embryo death is significantly correlated, r = 0.9432 (p<0.01). In the presence of 0.11mM EDTA, development of blastocysts and hatching or hatched blastocyst was higher in groups supplemented with iron, often significantly, in some groups supplemented with 2 to 20µM of iron (p<0.05).
Conclusion
It has been demonstrated there is a critical requirement for iron in embryo culture medium. Iron present in embryo culture medium is barely sufficient but blastocysts developed supposedly due to presence of sufficient stores of cellular iron of maternal origin. Significant improvement in embryo development occurs with iron supplementation. Iron depletion in culture medium adversely affects embryo development. Ferric iron is toxic at 8 µm concentration but not toxic up to 20 µm in presence of 0.11 mm EDTA. Iron tolerance levels range from 0.4 to 20 µm+0.11 mm EDTA and the optimal level is 2 µm+0.11 mm EDTA.

Introduction

In vitro generation of human embryos is a critical component of assisted reproduction technology (ART) and fertility treatment. It is well–recognized embryos of good quality have a better chance of implantation and viability (Sunde et al., 2016; Chronopoulou and Harper, 2015). The in vitro conditions are less optimal for human embryo development (Schramm and Bavister,1996). It follows that the most crucial factor in ART is the provision of culture milieu that minimize intracellular trauma or stress (Gardner and Lane, 2003) comparable to the natural conditions so as to allow generation of high quality, viable embryos.

Metallic ions such as iron or copper if present in the culture medium may lead to increased

ROS generation (Guerin et al., 2001). Bedaiwy et al. reported that increased levels of ROS in the culture medium is correlated with impaired blastocyst development, cleavage, and fertilization, as well as increased fragmentation rates, all of which will affect ART treatment adversely (Bedaiwy et al., 2004). Preimplantation embryos are extremely sensitive to conditions that induce oxidative stress (Fujii, et al., 2005).

During embryo culture in vitro reactive oxygen species (ROS) are generated (Paszkowski and Clarke, 1996; Lee et al., 2012). In the natural environment the levels of ROS are usually effectively controlled [Guerin et al., 2001] and are also utilized for oocyte maturation (Miyazaki et al., 1991] and sperm capacitation (de Lamirande et al.,1998). On the contrary perturbations to the embryo culture system in vitro that result in elevated levels of ROS are harmful and associated with poor or arrested embryo development [Guerin et al., 2001; Bedaiwy et al., 2004; Agarwal et al., 2003).

According to a report by Gutteridge, most laboratory chemicals and reagents contain trace (1.6 to 19.4 µm) amounts of iron as contaminants (Gutteridge, 1987). Embryo culture medium is made of chemicals. Following its preparation the embryo culture medium is subsequently supplemented with serum proteins prior to use. This suggests that iron could be present in all culture media as a contaminant even if it was not intentionally introduced by the originators of the culture media.

A recent report by Morbeck and coworkers (Morbeck et al., 2014) that compared the constituents of various commercial ART human embryo culture medium noted the level of contaminant iron to vary from 0 to 57 µg/l. A high level of iron of 57 µg/l was observed in one medium only as opposed to 0 to 11 µg/l in remaining media; raising the concern that there is no control over the level of contaminant iron introduced into the medium, and that such high levels of iron in the media may lend itself to deleterious ROS generation which could subsequently adversely affect the quality of embryos generated and the treatment outcome. For the purpose of clarity contaminant iron is defined as the concentration of detectable iron present in the embryo or cell culture medium when the culture medium was manufactured without iron.

In spite of its critical role in mammalian physiology and metabolism there is a paucity of data on the effect of iron on preimplantation embryonic development. A search of the literature turned up only three reports (Nasr-Esfahani et al., 1990; Ali, 2000; Naes et al., 2017) which were reported about a decade from each other indicating a lack of inquiry into the effects of iron on embryo development.

GMP-manufactured embryo culture media for human therapeutic ART are made up of highly purified ingredients and purified water, because of which the amounts of iron in the culture medium should, logically, be very negligible unless iron is supplemented in the culture medium. It is therefore crucial to determine whether iron in the embryo culture medium is essential for preimplantation development. Interestingly viable human embryos do develop in culture media that are made up without iron supplementation indicating that the amounts of iron introduced into the culture milieu as a contaminant through donor proteins and possibly cytoplasmic stores of maternal origin are sufficient for embryo development.

In some commercial culture media donor protein supplement is supplied separately and is added into the culture medium just prior to use. During routine quality control procedures the iron content of two commercial media (Irvine Scientific, USA and Cellcura ASA, Norway) was found to be 0 µm (Idris and Ali, unpublished observations, 2014) in the base medium but increased to 2.0 µm after addition of its donor protein supplements to the former medium (Irvine Scientific, USA) indicating iron most likely gets into the medium as a contaminant of donor proteins. In light of available evidence and the report of Morbeck and coworkers (Morbeck et al., 2014) it is speculated that high embryo fragmentation rates and the consequent poor ART treatment outcome occasionally seen with some batches of human embryo culture media may be due to high levels of contaminant iron in affected batches?

The main objective of this study is to determine (i) whether iron is critical for embryo development in vitro; (ii) the optimal concentration, (iii) tolerance and (iv) toxic levels

of iron for embryos in vitro; (v) the effect of iron depletion on embryo development and, (vi) whether iron present in embryo culture media as a contaminant is sufficient for embryo development?

Materials and Methods

Ethics approval
This study was approved by the Institutional Animal Experimentation Ethics.

Mouse embryos
Swiss outbred (SO) mice were used to generate embryos. The mice were acclimatized for 3 weeks and maintained on a light:dark period of 14:10 hrs with the light period starting at 4 pm. This was to enable the authors perform this study after hours, after completion of their daily clinical work commitments. The methodology for generation and recovery of mouse embryos were based on the methods described by Hogan and coworkers (Hogan et al., 1994). The SO mice are also known as the Quakenbush strain (Qs). They are a blocking strain (Du and Wales, 1993) and were used in this study for their high sensitivity to environmental perturbations. The sibling embryos recovered from individual mice were pooled and randomly apportioned to individual treatments before re-commencing embryo recovery on the remaining mice.

Culture medium and embryo culture
The culture medium used was the Whitten's medium. Whitten's medium was made up according to the formulation described by Whitten (Whitten, 1971) which was further improved in 1993 by Whitten and colleagues. Osmolality was adjusted to 285 milli osmols. Embryos were cultured in this medium in an atmosphere of 5 % carbon dioxide in air.

Visual observations of embryo development were performed on a daily basis to monitor the development of embryos so as to elucidate the involvement of iron on embryo development in vitro. The quality of mouse embryo development was evaluated as previously described (Hogan et al., 1994). It is well documented the in vivo development of mouse embryos to the blastocyst stage takes about 3.5 to 4.0 days post fertilization (Whitten and Dagg, 1961). In comparison to in vivo development it is well recognized and is common knowledge that in vitro development of mouse embryos is slightly slower compared to in vivo development such that blastocysts may only appear on mid-day 4 or day 5 in vitro. Due to the postponement of nearly 8 hours in the light:dark period in the present study the embryo development will be slower in development commensurate to the delay in fertilization that occurred due to the delay in the light:dark period introduced by the investigators. Thus day 2 embryos are defined as 2- and 4-celled embryos; day 3 embryos, as 4 to 8-cell embryos; day 4 embryos, morula, compacting and compacted morula; day 5 embryo, compacted morula, early blastocyst and blastocyst; and day 6 embryos, expanded, hatching and hatched blastocysts. The end point was the development of day 6 embryos.

Specific experiments
Experiment 1: Effect of iron depletion on embryo development
This experiment examined the effect of removal of iron from the culture medium on embryo development. The removal of iron from the culture medium will be effected by supplementing the medium with graded amounts of the high affinity iron chelator, DES. The objective is two-pronged, (i) depletion of iron in the medium and (ii) determine the optimal concentration of DES that can be tolerated by the mouse embryo. 1-cell SO sibling zygotes were challenged with various concentrations of DES in 4 separate experiments. The culture period extended over a period of 6 days. The concentrations of DES used were: Experiment 1a: 0, 20, 40, 60, 80, 100 µm, performed in 2 replicates involving n=119 zygotes; Experiment 1b: 0, 4, 8, 12 and 16 µm, performed in 2 replicates, n=146 zygotes; Experiment 1c: 0, 1, 2, 3, 4 µm, 4 replicates n=305 zygotes, Experiment 1d: 0, 200,400, 600, 800 nm, performed in 6 replicates using n = 329 S.O. zygotes.

Experiment 2: Effect of added ferric iron on embryo development
This experiment was designed to elucidate the toxicity levels, tolerance and optimal concentration of ferric ($FeCl_3$) iron for mouse embryo development. A stock solution of ferric iron (Ferric Chloride Hexahydrate; Sigma Aldrich, USA, Cat. No. F2877; Linear Formula: $FeCl_3 \cdot 6H_2O$; Molecular Weight: 270.30; CAS Number: 10025-77-1) was made in the culture

medium. 1-cell SO mice were cultured in culture medium containing graded amounts of ferric chloride. The levels of ferric iron tested ranged from 400 nm to 60 µm performed in separate experiments (Experiment 2a: 5, 10, 15 and 40 µm; Experiment 2b: 2, 4, 6, 8,10 µm; Experiment 2c: 400, 600, 1200, 1600 nm). Experiment 2 was undertaken in a manner similar to Experiment 1. The numbers of replicates were three per experiment.

Experiment 3: Effect of added ferric iron in the presence of EDTA on embryo development
Experiment 3 was identical to experiment 2 except that the culture medium was further supplemented with EDTA. The possible role of 0.11 mm EDTA [25] in ameliorating the potential deleterious effects of iron that is present in the culture medium as a contaminant and the supplemented graded amounts of ferric iron on embryo development was determined. The levels of ferric iron tested ranged from 400 nm to 20 µm performed in separate experiments (Experiment 3a: 0, 5, 10, 15 and 20 µm; Experiment 3b: 2, 4, 6, 8,10 µm; Experiment 3c: 400, 600, 1200, 1600 nm). Experiment 3 was undertaken in a manner similar to Experiment 1. The numbers of replicates were three per experiment.

Statistical analysis
Statistical comparisons were performed with the use of the two by two tables, Pearson's Chi-square and Pearson's correlation using the software *Statistix*TM.

Results

Experiment 1: Effect of iron depletion on embryo development

Experiments 1a to 1c: The effect of Desferrioxamine on embryo development in vitro (µm levels).
Chelation of iron by DES at the 1 to 200 µm concentrations resulted in the death of S.O. zygotes (n = 471 zygotes used; for these experiments involving 3 replicates). The 1-cell zygotes employed arrested at the zygote stage or 2-cell stage in the presence of 2 to 100 µm of DES The control embryos in these experiments cultured under similar conditions but without DES developed to the day 6 blastocyst stages

(76.5 %; n = 17; 2 replicate). The study was not continued when at least two replicates were found to confirm the levels of DES used was toxic to embryos. Of these concentrations 1 µm DES alone supported blastocyst development but this was statistically insignificantly lower than the development of blastocysts in the control group (p = 0.0584). Figs.1a&b shows trends in the development of blastocysts and death of mouse embryos when exposed to various concentrations of DES up to 2.0 µm. The mortality of embryos was insignificant until 1.0 µm but was significantly high at 2.0 µm concentration (p <-0.01) and beyond.

Experiment 1d: The effect of DES on embryo development in vitro (nm levels).
The experiment was repeated with reduced levels of DES at the nanomolar concentrations of 200 to 800 nm. The development of embryos in all treatments was similar to the control group of sibling embryos (Table 1). Chi-square analysis showed no difference in the trend of development of embryos from day 1 to day 6 in the absence or in the presence of 200 to 800 nm of DES (p = 0.9999). Comparison of the trend in the daily development of embryos when compared individually between treatments and the control embryos showed no trend and that all treatments were comparable to the control group on a daily basis (0.9988; 0.9992; 0.8423; 0.9922) from the lower to higher concentrations of DES. The development of embryos on day 6 (expanded, hatching and hatched blastocysts) were similar for all treatments when compared to the control group (p = 0.9991, 0.9972, 0.6569 and 0.2905). Chi-square for trend on day 6 was 0.424 which was not significant (P = 0.5147) indicating no trend with increasing concentrations of DES.

Experiment 2: Effect of ferric iron supplementation on S.O. embryo development.

Experiment 2.1: Effect of Iron supplementation on embryo development in vitro (20 to 60 µm)
Ferric iron was toxic at the 20 to 60 µm levels (n=298 S.O. zygotes; 3 replicates).

Experiment 2a: Effect of Iron supplementation on embryo development in vitro (5 to 20 µm)

Embryotoxicity of ferric iron was discernible at the 20 μm level (Table not shown) by day 3 of culture. Other treatments also demonstrated transitional embryo toxicity on day 4 at the 5 μm, on days 4 and 5 at the 10 μm and on day 5 at the 15 μm levels. However a comparison of the trend in the daily development of embryos when compared between individual treatments using Pearson's Chi-square analysis showed the control group to be similar to all treatments except for the 20 μm concentration (p = 0.9653; 0.8672; 0.9091; 0.0245) respectively from the lower to higher concentrations of ferric iron used. The transitional embryo toxicity demonstrated by individual treatments 5 μm, 10 μm and 20 μm beginning from day 4 is a crucial finding, and a significant observation although statistically the trend in the development of embryos in culture appear similar to the control for all treatments except 20 μm.

Experiment 2b: Effect of Iron supplementation on embryo development in vitro (2 to 10 μm)
Embryotoxicity of ferric iron was discernible at the 8 μm level (Table 2a). A comparison of the trend in the daily development of embryos when compared between individual treatments using Pearson's Chi-square analysis showed the control group to be comparable to all treatments except for the 10 μm concentration (p = 0.9432; 0.9903; 0.9747; 0.2048; 0.0227) respectively from the lower to higher concentrations of ferric iron.

Experiment 2c: Effect of iron supplementation on embryo development in vitro (400 to 1600 nm)
Ferric iron supplementation at the 400 to 1600 nm levels had no impact on embryo development (Table 2b; replicates=3; total nos. of embryos =200). All values in the test groups were comparable to the control group. However the correlation "r" between iron supplementation and embryo death is significant, r = 0.7669 (p <0.05) indicating a trend towards embryotoxicity with increasing concentrations of ferric iron. (Total nos. of embryos = 200; replicates=3). Chi-square for trend on day 6 was 21.107 which was highly significant (P <0.0001) indicating a trend towards non-viability of embryos with increasing concentration of iron.

Experiment 3: Effect of added ferric iron in the presence of EDTA on embryo development

Experiment 3a: Effect of added ferric iron (5 to 20 μm) in the presence of 0.11 mm EDTA on embryo development
Table 3a shows EDTA ameliorating the deleterious effects of supplemented ferric iron. A comparison of the trend in the daily development of embryos showed the control group was comparable to all treatment groups indicating no difference between the various groups tested from lower to higher concentrations of ferric iron.

Experiment 3b: Effect of added ferric iron (2 to 10 μm) in the presence of 0.11 mm EDTA on embryo development
A comparison of the trend in the daily development of embryos showed the control group was comparable to all treatment groups indicating no difference between the various groups tested from the lower 2μm to higher concentrations (10 μm) of ferric iron (Table not shown). The development of embryos on days 5 and 6 were comparable in all groups.

Experiment 3c: Effect of added ferric iron (400 to 1600 nm) in the presence of 0.11 mm EDTA on embryo development
A comparison of the trend in the daily development of embryos showed the control group was comparable to all treatment groups indicating no difference between the various groups tested (Table not shown).

Blastocyct development after supplementation with ferric iron in the absence and presence of 0.11 mm EDTA

Figs. 2 show the comparative graphic representation of blastocyst development after ferric iron supplementation in the absence or presence of 0.11 mm EDTA. Blastocyst development in all treatments were comparable to controls except for embryos treated with 8 μm and 10 μm concentrations [Figs 2a(i) and 2b(i)] (and beyond) of ferric iron in the absence of EDTA. Ferric iron could be toxic at levels as low as 8 μm in the absence of EDTA. However blastocyst development was comparable to the control values (p >0.05) at the highest level of ferric iron tested (20 μm) in the presence of 0.11 mm EDTA. All embryos in the 2.0 μm

Table 1: Effect of iron depletion by chelation with desferrioxamine on mouse embryo development in vitro

Description	Concentration of desferrioxamine (nM) and % development of embryos					
	0	200	400	600	800	
Day 1 (1-cell embryos)	n=76	n=75	n=76	n=75	n=75	
Day 2 (2 to 4-celled embryos)	100	100 (ns)	100 (ns)	100 (ns)	100 (ns)	
Day 3 (4 to 8-celled embryos)	96.1	100 (ns)	100 (ns)	100 (ns)	100 (ns)	
Day 4 (Morula, compacting and compacted morula)	69.7	68.0 (ns)	68.0 (ns)	66.7 (ns)	70.7 (ns)	
Day 5 (Compacted morula, early blastocyst and blastocyst)	56.6	50.7 (ns)	56.0 (ns)	40.0 (ns)	57.3 (ns)	
Day 6 (Expanded, hatching and Hatched blastocysts)	42.1	42.7 (ns)	46.7 (ns)	37.3 (ns)	50.7 (ns)	
[+]p-value (Trend in daily development for individual concentrations of DES)		0.9988	0.9992	0.8423	0.9922	

ns= Not significant (p>0.05); s=significantly different (p <0.05) + Treatments compared individually with control.

Chi-square for trend on day 6 = 0.424 (P = 0.5147)

Table 2a: Effect of ferric iron on mouse embryo development in vitro

Description	Concentration of iron (µM and % embryo development)					
	0	**2**	**4**	**6**	**8**	**10**
Day 1 (1-cell embryos)	n=40	n=39	n=40	n=40	n=40	n=41
Day 2 (2 to 4-celled embryos)	95.0	100 (ns)	97.5 (ns)	100 (ns)	97.5 (ns)	92.7 (ns)
Day 3 (4 to 8-celled embryos)	92.5	92.3 (ns)	90 (ns)	95 (ns)	67.5(s)	46.3 (s)
Day 4 (Morula, compacting and compacted morula)	87.5	84.6 (ns)	77.5 (ns)	80.0 (ns)	50.0 (s)	43.9 (s)
Day 5 (Compacted morula, early blastocyst and blastocyst)	82.5	79.5 (ns)	70.0 (ns)	67.5 (ns)	45.0 (s)	39.0 (s)
Day 6 (Expanded, hatching and Hatched blastocysts)	77.5	56.4 (s)	67.5 (ns)	67.5 (ns)	40.0 (s)	26.8 (s)
[+]***p*-value** (Trend in daily development for individual concentrations of DES)		0.9432	0.9903	0.9747	0.2048	0.0227

ns= Not significant (p>0.05); s=significantly different (p <0.05) + Treatments compared individually with control.

Chi-square for trend on day 6 = 21.107 (P <0.0001)

Figs.1a,b: The effect of desferrioxamine on mouse embryo development
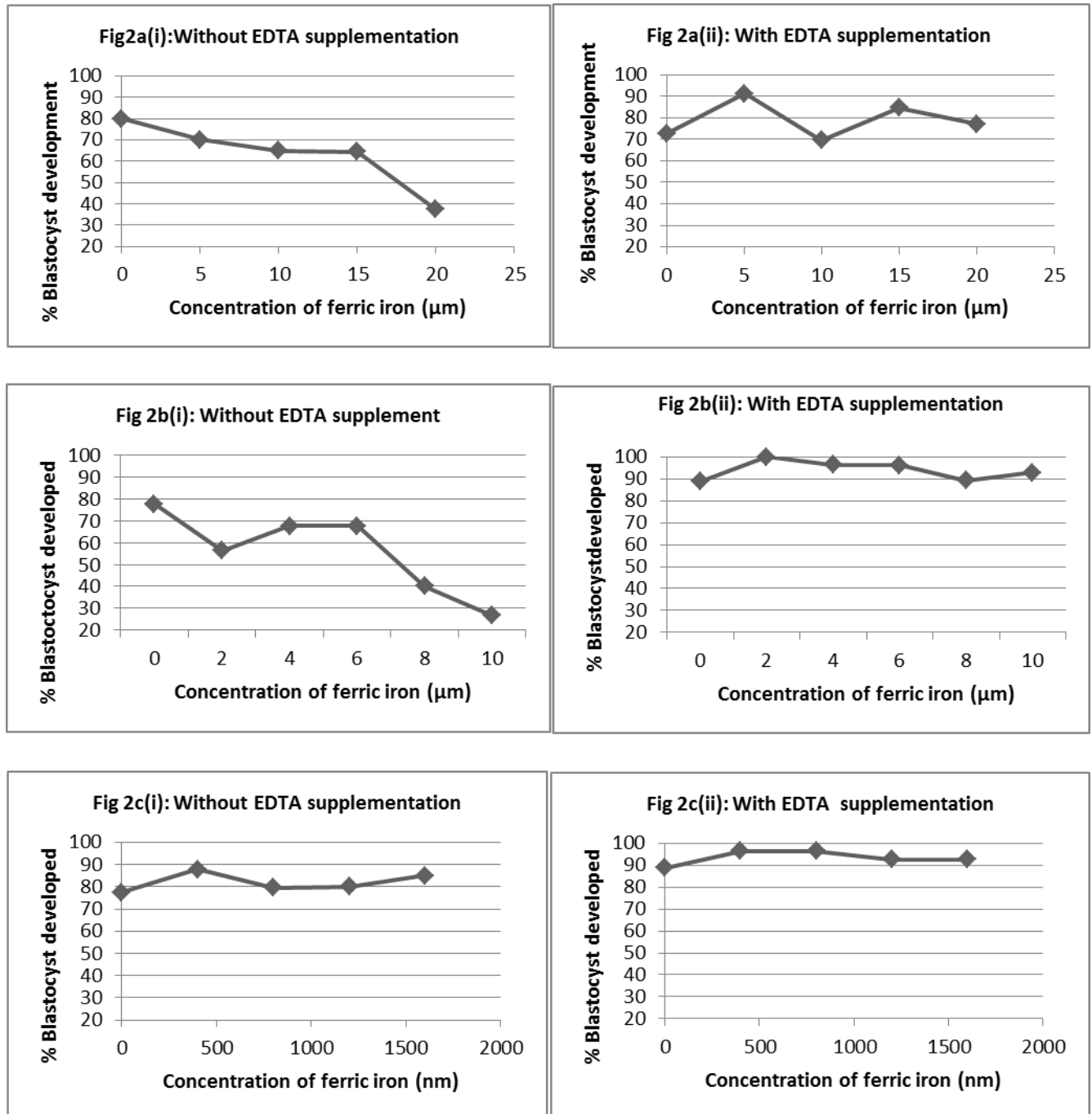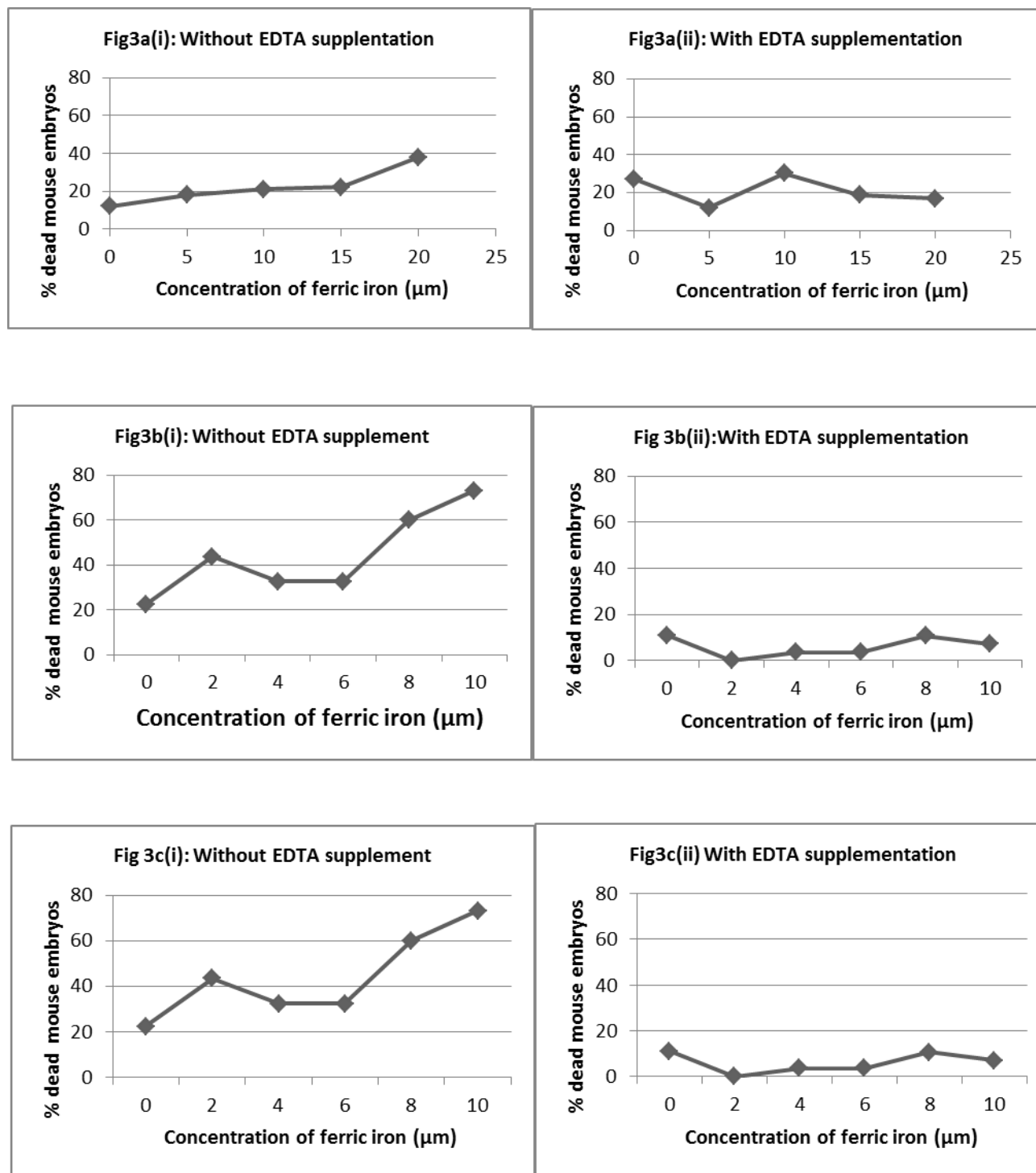

Fig.1a: Per cent blastocyst development when exposed to desferrioxamine

Fig.1b: Per cent of mouse embryo mortality after exposure to desferrioxamine
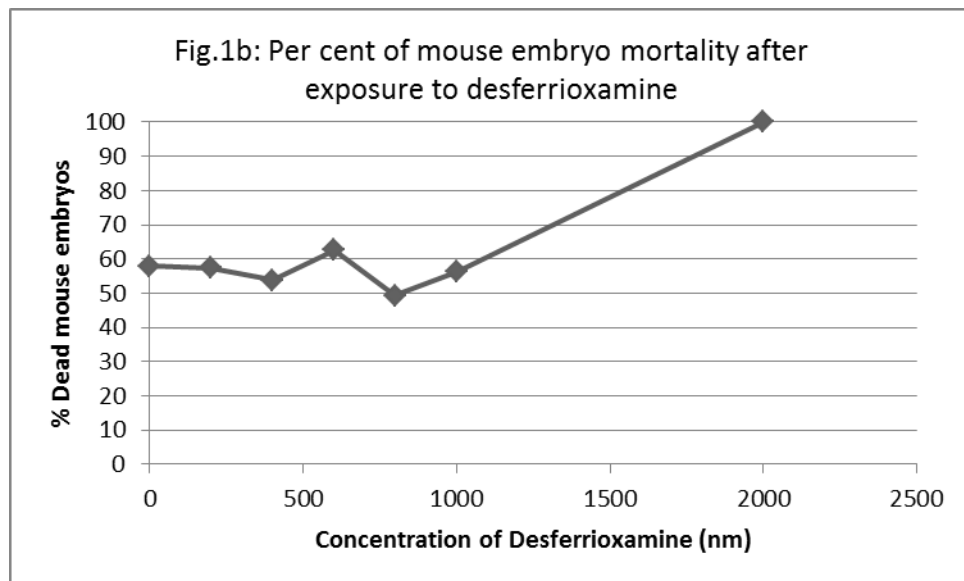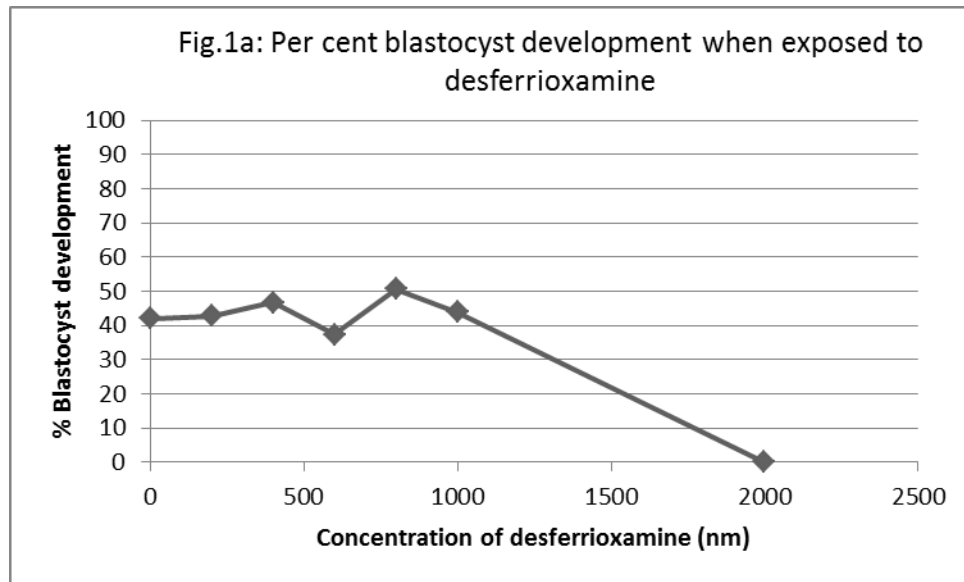
Table 2b: Effect of ferric iron (nM) on embryo development in vitro

Description	Concentration of iron (nM) and % embryo development					
	0	400	800	1200	1600	
Day 1 (1-cell embryos)	n=40	n=41	n=39	n=40	n=40	
Day 2 (2 to 4-celled embryos)	95.0	92.7 (ns)	97.4 (ns)	97.5 (ns)	97.5 (ns)	
Day 3 (4 to 8-celled embryos)	92.5	90.2 (ns)	92.3 (ns)	92.5 (ns)	87.5 (ns)	
Day 4 (Morula, compacting and compacted morula)	87.5	87.8 (ns)	89.7 (ns)	82.5 (ns)	85.0 (ns)	
Day 5 (Compacted morula, early blastocyst and blastocyst)	82.5	87.8 (ns)	82.1 (ns)	80.0 (ns)	85.0 (ns)	
Day 6 (Expanded, hatching and Hatched blastocysts)	77.5	87.8 (ns)	79.5 (ns)	80.0 (ns)	85.0 (ns)	
+p-value (Trend in daily development for individual concentrations of DES)		0.9970	1.000	0.9998	0.9988	

ns= Not significant (p>0.05); s=significantly different (p <0.05) + Treatments compared individually with control.

Chi-square for trend on day 6 = 0.134 (P = 0.7140)

Table 3a: Effect of added ferric iron in the presence of 0.11 mM EDTA on embryo development

Description	Concentration of Iron (µM) and percent embryo development					
	0	5	10	15	20	
Day 1 (1-cell embryos)	n=33	n=34	n=33	n=33	n=35	
Day 2 (2 to 4-celled embryos)	90.9	97.1 (ns)	100.0 (ns)	100.0 (ns)	100.0 (ns)	
Day 3 (4 to 8-celled embryos)	78.8	91.2 (ns	84.8 (ns)	90.9 (ns)	85.7 (ns)	
Day 4 (Morula, compacting and compacted morula)	78.8	91.2 (ns	84.8 (ns)	90.9 (ns)	85.7 (ns)	
Day 5 (Compacted morula, early blastocyst and blastocyst)	72.7	91.2 (ns	69.7 (ns)	84.8 (ns)	77.1 (ns)	
Day 6 (Expanded, hatching and Hatched blastocysts)	72.7	91.2 (ns	69.7 (ns)	84.8 (ns)	77.1 (ns)	
[+]**p-value** (Trend in daily development for individual concentrations of DES)		0.9877	0.9984	0.9979	0.9998	

ns= Not significant (p>0.05); s=significantly different (p <0.05) + Treatments compared individually with control.

Chi-square for trend on day 6 = 0.00699 (P = 0.9334)

Figs. 2: Comparative Per cent mouse blastocyst development after ferric iron supplementation in the absence or presence of 0.11mM EDTA

Fig2a(i):Without EDTA supplementation

Fig 2a(ii): With EDTA supplementation

Fig 2b(i): Without EDTA supplement

Fig 2b(ii): With EDTA supplementation

Fig 2c(i): Without EDTA supplementation

Fig 2c(ii): With EDTA supplementation

Figs. 3: Comparative per cent mortality of mouse embryos after culture with ferric iron in the absence or presence of 0.11 mm EDTA

Fig3a(i): Without EDTA supplentation

Fig3a(ii): With EDTA supplementation

Fig3b(i): Without EDTA supplement

Fig 3b(ii): With EDTA supplementation

Fig 3c(i): Without EDTA supplement

Fig3c(ii) With EDTA supplementation

treatment developed to the blastocyst stage in the presence of EDTA and serum proteins.

Trends in toxicity and mortality of mouse embryos following supplementation with ferric iron in the absence or presence of EDTA.

3.5.1. Figs 3 show a comparative graphic representation of embryonic mortality following supplementation with ferric iron in the absence or presence of 0.11 mm EDTA. The levels of ferric iron that appear to contribute to embryonic death was discernible at the levels of 8 µm and beyond in the absence of EDTA. In Experiments 2a and 2b the correlation "r" between iron supplementation and embryo death is significant, [r = 0.9214, p <0.02 and r = 0.8445, p <0.02 respectively] but was insignificant at concentrations below 800nM [r = -0.2662; (p >0.05)] indicating that the embryo toxicity of ferric iron increases progressively with concentration in the absence of EDTA. Chi-square for trend on day 6 of culture was 21.107 which was significant (p <0.0001) further proving a trend towards non-viability of embryos with increasing concentrations of ferric iron. EDTA appears to have a protective effect on mouse embryo viability nullifying the toxicity of ferric iron on embryo development in vitro up to the highest level (20 µm) of ferric iron tested. Of interest is the 0 % embryo mortality in the 2.0 µm treatment in the presence of EDTA and proteins.

Discussion

Iron is among the most ubiquitous element on the planet. It is therefore inevitable that iron play important and critical roles in biological processes including cellular metabolism. In mammals iron is a critical mineral required for numerous cellular processes Hentze et al., 2004; Aisen et al., 2001).

Studies have proven the deleterious effect of iron depletion on cell proliferation in culture that is well documented to affect G1/S and G2/M phases of the cell cycle effecting cycle arrests and apoptosis (Renton and Jeitner, 1996; Fu and Richardson, 2007; Nurtjahja-Tjendraputra, et al., 2007).

In the present study the use of DEX at the 1 µM concentration and above affected embryonic development in vitro. DEX is a potent chelator of iron.

The present study has also demonstrated that iron above certain concentration is very toxic for embryos when supplemented in culture media in the absence of chelators and antioxidants. This finding is compatible with previous reports (Abramczuk et al., 1977).

Embryos exposed to 2.0 µM of ferric iron +0.11 mm EDTA+serum proteins registered complete development of all embryos to the blastocyst stage with no (0 %) embryo mortality but this was statistically insignificant, though superior to the control values (88.9 % blastocyst development and 11.1 % embryo death). Although statistically there is no difference between the control and 2.0 µM but the total development of all embryos with no mortality suggest it to be the most optimal concentration. The lowest level that elicited a positive response under similar conditions was 0.4 µM. In view of the critical requirement for iron for cellular processes the authors recommended ferric iron augmentation be maintained between 0.4 to 2.0+0.11 mm EDTA which must be performed with considerable prudence without exceeding the latter concentration

The present investigation suggest the amount of iron present in embryo culture medium is just barely sufficient to meet the metabolic needs of the embryo and that prudent supplementation of the non-toxic levels of iron in presence of EDTA does improve culture conditions. Current findings show iron supplementation in the order of 0.4 to 20 µM in the presence of 0.11 mm EDTA to be beneficial for embryonic development.

Considering the prevalence of batch variation in the amount of iron that may be present as a contaminant in the culture medium (ranging from very low to very high levels (Fujii et al., 2005; Morbeck et al., 2014) (1.6 to 19.4 µm (Fujii et al., 2005); 0 to 57 µg/l, combined with the suggestions of presence of maternal stores of iron in the oocyte (Ali, 2000; Naes et al., 2017) it appears prudent and safer to either not supplement the human embryo culture medium with iron or to use iron with utmost prudence the

lowest level of iron that supported the development of blastocysts in the presence of EDTA in the embryo culture medium provided and the embryo culture media is deficient in iron.

Depletion of iron in cells typically results in a G1/S arrest, (Brodie et al., 1993; Lucas et al., 1995] indicating that this metal is essential for cell cycle progression, growth and division (Kwok et al., 2002; Le and Richardson, 2002; Richardson, 2005; Steegmann-Olmedillas, 2011). Under some conditions of iron depletion, a G2/M arrest has also been identified (Renton and Jeitner, 1996]. It is clear iron plays a crucial role in cell metabolism.

It was earlier postulated that the amount of maternal iron stores present in the oocyte and thence in the embryo following fertilization, and the amounts of contaminant iron from chemicals and the donor proteins used in the preparation of the embryo culture medium probably provides barely sufficient iron to support embryonic development (Ali., 2000). Indeed the findings of Morbeck and coworkers (Morbeck et al., 2014) on currently available commercial human embryo culture media showed iron levels to generally range from 0 to 11 µg/l with the exception of one medium that was found to contain 57 µg/l of iron. It was noted iron was absent in at least 2 media that contained donor proteins or serum products. This iron-deficient state contrary to expectation did not affect the quality of embryos generated which is perplexing. There is a large volume of data that is common knowledge accrued over the last few decades of human embryo culture that has shown excellent embryo development and high embryo viability in embryo culture media now shown to be deficient in or devoid of iron. It therefore appears plausible that the suggestion of Ali (Ali, 2000) that adequate iron stores within the embryonic cytoplasm of maternal origin exists often together with contaminant iron in the medium; these sources of iron appear sufficient to enable embryonic cell division proceed unhindered.

The present finding plus previous observations of S.K. Idris and J. Ali (unpublished observations; University of Malaya, 2014), and that of Ali and coworkers (Ali et al., 2014) noted commercial embryo culture media such as PFM, Cellcura ASA (Ali et al., 2014) and, Global

Medium, IVFOnline, USA, and G1 Medium, Vitrolife, Sweden; Idris and Ali, 2014) had zero levels of iron. The findings of Morbeck and coworkers (Morbeck et al., 2014) were similar for a number of commercial embryo culture media. However all these media supported the development of viable human embryos unabated. This curious fact that human preimplantation embryos developed in culture milieu deficient in iron further strengthens the speculation intracellular iron of maternal origin exists in sufficient amounts in the embryonic cytoplasm to enable preimplantation development unhindered even if the milieu of the embryo is deficient in iron.

Conclusion

Based on present findings supplementation of iron in the order of 0.4 and 20 µM in presence of 0.11 mm EDTA proved beneficial for embryo development in vitro. Nevertheless, considering the deleterious nature of iron and the proven development of blastocysts in iron deficient media its supplementation in embryo culture media is recommended if an augmentation is prudently retained between 0.4 to 2.0 µM levels without exceeding the 2.0 µM level in the presence of EDTA. It has been demonstrated iron is crucial for embryo development. Although the amount of this element present in embryo culture medium is just barely sufficient to meet the metabolic needs of the embryo nevertheless preimplantation development progressed unhindered raising speculation of the presence of sufficient stores of cellular iron of maternal origin in the embryos in amounts sufficient for cellular activities. Ferric iron is toxic above the 8 µM level in the absence of EDTA.

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