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Original article

Impact of elemental iron on human spermatozoa and mouse embryonic development in a defined synthetic culture medium

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ABSTRACT

There is a paucity of studies on effect of iron in embryo culture procedures. This study aims to ascertain the optimal, tolerance and toxic levels of iron in a protein-free embryo culture medium (PFM) to determine the effect of iron on embryonic development. The application of PFM in assisted reproductive technologies (ART) is intended to eliminate disease transmission and improve ART treatment outcome. The optimal, tolerance and toxic levels of iron on human spermatozoa and mouse embryos were determined by challenging them with different levels of iron (ferric iron; Fe^{+3}). Human normozoospermic semen samples (n = 24) and days 1–4 Quakenbush Special (Qs) mouse embryos (n = 1160) were incubated in PFM supplemented with different concentrations of Fe^{+3} over different periods of time. 2.0 μg/mL (35.8 μM) of Fe^{+3} was the optimal level of Fe^{+3} for human spermatozoa with a tolerance range of 0.5–2 μg/mL; whereas a level of 0.11 μg/mL (2 μM) of Fe^{+3} was the optimum for day 2 embryos. Levels of ferric iron at 0.11 to 2.8 μg/mL appear to enhance spermatozoa motility, preserve its DNA integrity and possibly increase percentage of blastocysts developed but levels of ferric iron >16 μg/mL is hazardous for both spermatozoa and embryos. In spite of beneficial effects of iron it is premature to recommend its supplementation in embryo culture media because of the known deleterious nature of iron and the paucity of toxicological data. Toxicological studies must be performed following which it can be decided whether it is safe to consider iron as a supplement in human embryo and spermatozoa culture media.

1. Introduction

Iron is crucial for life. It performs key roles in biological processes which includes electron transport, energy production, gas sensing, DNA synthesis, cell division and proliferation [1–3]. Iron and its compounds are basically not toxic for the human organisms [4] except when delivered in high doses. A wide range of disturbances can occur following prolonged intake of high doses of iron, or in some pathological conditions such as disorders affecting the regulative absorption mechanism [5]. At the other end of the spectrum iron deficiency minimizes the activity of iron-containing and iron-dependent enzymes [6]. The role of iron has been highlighted by some studies especially in spermatogenesis, spermatozoa metabolism [7] and the iron-transporting proteins in key ovarian cells. It is documented iron has a direct regulator role in three mammalian gene expressions [8], of which two have an impact on male reproduction. Moreover, transferrin and its receptor were documented in several studies to be present in granulosa cells and oocytes [9–11]. More recently, it has been reported that granulosa cells can synthesize transferrin; which may be translocated to the oocytes [11]. The total iron content of the human seminal plasma is estimated to be 2.59 ± 0.21 mg/kg [12] which is thought to be crucial to preserve the motility and viability of spermatozoa after ejaculation and to retain it functions in vivo. Another study by Marzec-Wróblewska et al. [4] reported the presence of 18.265 ± 8.599 mg/kg of iron in semen which appears dramatically different to the previous report by Slivkova et al. [12]. In general, the amount of iron in the human body under normal conditions ranges around 3–4 g [13]. While, the normal range of iron in human plasma is 0.6–1.7 μg/ml which is equivalent to 10–30 μM [14].

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Of the recently developed embryo culture media, the synthetic protein-free medium (PFM) [15–17], has the ability to prevent disease transmission because it is devoid of added human serum albumin (HSA). HSA has the potential to be a source of pathogens. It is also the main source of iron in protein-containing embryo conventional culture media (CCM). As PFM is devoid of HSA it was anticipated to have very little iron as a contaminant in the medium. Mojic et al. [18] found that cell culture medium with iron at physiological levels of human plasma prevented the accumulation of hydrogen peroxide (H_2O_2) which is one of reactive oxygen species (ROS) involved in oxidative stress (OS) development. In addition it was documented low levels of iron in the order of $\leq$10 μmol/L $FeCl_2$ and $\leq$50 μmol/L $FeCl_3$ were able to activate bovine spermatozoa motility and stabilize oxidative balance [19,20]. Testicular atrophy, epididymal lesions, morphological changes in testes, and defect in spermatogenesis and reproductive performance, are problems resulting as a consequence of disproportionate levels of iron [21–23].

Moreover, OS plays a major role in the pathophysiology of infertility in females with iron overload. This OS is mainly caused by tissue injury due to overproduction of free radicals by secondary iron overload, alteration in serum trace elements, and alteration in antioxidant enzyme levels. Consequently, there is a rationale for iron chelation to eliminate the free iron species which, in this respect, act like antioxidants [24]. Polycystic ovary syndrome (PCOS) patients had iron excess and higher hepcidin levels, which are related with metabolic disturbance [25].

There are numerous reports on the influence of different elements on human spermatozoa but very few on the effects of supplemented iron on embryonic development in vitro. The present study aims to ascertain (i) the amount of iron (ferric chloride) needed for optimal human spermatozoa activity and mouse embryo development, (ii) the range of iron that can be tolerated by the spermatozoa and mouse embryos, and (iii) the concentration of iron at which its toxicity is expressed on spermatozoa and embryos in a protein-free culture media previously developed by one of us (JA) [15–17].

2. Materials and methods

2.1. IRB approval

This study was approved by the University of Malaya Medical Center Ethics Committee (IRB) Ref. No. 1198.52 for work with human semen samples. The work on mouse embryos was approved by the Institutional Animal Experimentation Ethics Committee with Ref. No. 2014-07-01/ONG/R/"JAMA for experiment using mouse embryos.

2.2. Methodology

2.2.1. Determination of the level of iron in HEPES PFM and PFM Media

Iron level in HEPES PFM was determined by flame atomic absorption spectrophotometer (AAS) (Perkin Elmer, Aanalyst 400). Sample preparation was performed according to Altekin et al. [25] with slight modifications. Sample was diluted in deionized water with 0.1 M of hydrochloric acid (HCL; Sigma Chemical Co, USA) with dilution factor; 1:10. Calibration was achieved by diluting the stock solution of Iron (III) Nitrate AAS Standard [1 mg/mL] (Ajax Finechem Pty Ltd, Australia) to 1, 2, and 3 mg/L. The iron level in PFM culture medium was determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent, 7500 Single Turbo System). Three readings were taken per replicate.

2.3. Effect of ferric iron on spermatozoa activity

2.3.1. Semen sample preparation and exposure to ferric iron

Normozoospermic semen samples were collected from 24 male patients undergoing semen assessment. Semen sample was collected by masturbation following 2–3 days of sexual abstinence. Semen analyses were carried out as per the recommendations of the World Health Organization (Semen Manual Year 2010) [26]. Following collection the samples were transported at ambient temperature (25–29 °C) from a donor hospital to the laboratory of the investigators which took approximately 20 to 30mins. Semen analyses were performed as per the methodology described in the Semen Manual of the World Health Organization [26] which included: semen volume, pH, sperm concentration, percentage motility and vitality.

Sibling spermatozoa from well-mixed samples were diluted in HEPES buffered PFM medium containing different concentrations of ferric (Fe^{+3}) in the order of 0, 0.5. 1.0, 1.5 and 2.0 μg/mL for studies to determine the optimal concentration and tolerance levels of iron; while studies to determine toxic levels of ferric iron utilized 0, 4.0, 8.0, 12.0, and 16.0 μg/mL of iron. The dilution was depended on the original spermatozoa concentration, with a dilution ratio of 1:20 or1:40 if spermatozoa concentration was very high (>100 million per mL) with HEPES buffered PFM, performed using 96-well plates (MTP, Greiner, Germany) as described by Trvda et al. over a period of 24 h at room temperature (22–25 °C). The following tests were performed at 1 and 20 h: (i) spermatozoa motility by using the Neubauer chamber, (ii) spermatozoa vitality test and the (iii) spermatozoa DNA fragmentation test.

2.3.2. Spermatozoa motility evaluation

The motility analyses of all treatments were performed using a modified Neubauer Chamber (Hawksley, Lancing, England) according to WHO semen analysis 2010 [24] using a phase contrast microscope (Olympus-CH2, Japan) at 20× magnification. Neubauer chamber was incubated for 10 min at 37 °C, then a 10 μmL of spermatozoa suspension was placed on it. The specimen was examined at 37 °C under a phase contrast microscope. Spermatozoa were scored as progressive, non-progressive, and non-motile.

2.3.3. Spermatozoa vitality evaluation

Spermatozoa vitality was ascertained by using the Spermatozoa VitalStain™ (Nidacon International AB, Sweden). This is a well-documented staining system used to determine the proportion of life and dead spermatozoa in a given specimen. The procedure was performed according to the instructions of the manufacturer. Each specimen was read until 200 spermatozoa have been evaluated per slide.

2.3.4. Sperm DNA fragmentation (SDF) test and DNA integrity

Sperm DNA fragmentation analyses and integrity was analyzed using the Halosperm® G2 Kit (Halotech DNA S.L., Spain) according to the instructions of the manufacturer. Sperm DNA integrity of each treatment was performed alongside a negative control. Treated spermatozoa were stained with Eosin dye and then Azure dye (both of which are included in Halosperm® G2 Kit). Spermatozoa with a halo of chromatin around the sperm head was considered as exhibiting DNA integrity, whilst the spermatozoa without halo were indicative of DNA fragmentation. A total of 200 treated spermatozoa were observed under bright field light microscope (Olympus-CH2, Japan) at 40× magnification Spermatozoa DNA Integrity level was calculated by dividing the number of spermatozoa (with Halo) by total number of spermatozoa × 100.