Cryobiology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Cryobiology

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

Effect of metformin on the fertilizing ability of mouse spermatozoa ☆

Michael J. Bertoldo [a], Edith Guibert [a], Pauline Tartarin [a], Vanaique Guillory [b], Pascal Froment [a,*]

[a] Unité de Physiologie de la Reproduction et des Comportements, Institut National de la Recherche Agronomique, UMR85, F-37380 Nouzilly, France
[b] INRA Centre Val de Loire, Pôle Santé Animale de Tours, Plate-Forme d'Infectiologie Expérimentale, F-37380 Nouzilly, France

ARTICLE INFO

Article history:
Received 8 January 2014
Accepted 7 February 2014
Available online xxxx

Keywords:
Metformin
Mouse sperm
Anti-oxidant
Motility
Cryopreservation
IVF

ABSTRACT

Numerous antioxidants have been added to cryopreservation media with varied success. The biguanide, metformin, commonly used for the treatment of type II diabetes, possesses properties impacting metabolism control that have not been yet assessed in cryopreservation protocols. The aim of this experiment was to; (i) determine the effect of metformin on fresh spermatozoa properties; and (ii) to assess positive or negative effects of metformin in post-thaw function and fertilizing capacity of mouse spermatozoa when used in cryopreservation media. The experiments have shown that the presence of metformin in fresh semen did not induce negative effects on spermatozoa quality, except a slight reduction in sperm motility at 5000 μM metformin. However, when metformin was included in a cryopreservation protocol, an improvement in the fertilization rate and a reduction in the percentage of abnormal zygotes after *in vitro* fertilization was observed. In conclusion, metformin did not affect sperm quality at low concentrations (50 μM), but its presence in the cryopreservation media could represent a benefit to improve the quality of frozen semen.

Introduction

The ability to cryopreserve spermatozoa has been practiced for a considerable amount of time [11,23], and is the most convenient technique for the long-term storage of spermatozoa. It is a valuable technique used to conserve precious genetic material for domestic and endangered species and manage infertility in humans. However despite the advancements made over the years, in most species, the post-thaw quality and function of spermatozoa is impaired when compared to fresh sperm [11,29,45]. Cryopreservation causes permanent damage to spermatozoa such as loss of motility, reduced DNA integrity, damage to the acrosome and plasma membrane, and apoptosis [16,30,31,38,47]. The reasons for these negative effects are generally accepted to be caused by cold shock, oxidative stress and hypertonic damage [29,44]. In addition, it is becoming increasingly accepted that even when membrane intact, motile spermatozoa are recovered after thawing, these cells are functionally distinct from fresh sperm [45]. Spermatozoa are particularly vulnerable to oxidation of their lipid plasma membranes due to the composition of fatty acids in the membrane and the relative inability to combat against oxidative stress. As the sperm cell is a highly specialized cell which has the capacity to be motile and to fuse with the oocyte after the acrosome reaction, the quality of membrane and efficiency of mitochondria are crucial for proper spermatozoa activity.

Besides the cryopreservation protocol implemented, the extender composition and the nature of the external cryoprotectant compounds are of critical importance for spermatozoa survival following cryopreservation [6,12,36]. Various antioxidants have been added into the cryopreservation media and have been observed to improve spermatozoa function such as motility and membrane integrity in numerous species [5,6,8,12,17]. Among the family of molecules with antioxidant properties [5,10,17], metformin has not yet been tested on spermatozoa function following cryopreservation. Metformin is a molecule of the biguanide family, and has the ability to decrease reactive oxygen species [15,26,28,33] and to activate the transcription factor Nrf2, resulting in increased expression of antioxidant genes [32]. At the cytoplasmic level, metformin is able to lower the activity of mitochondrial complex I, which results in less reactive oxygen species. Although metformin has been in use for 50 years, it is only since the last decade that the mechanism of action of metformin began to be understood. Indeed, Zhou et al. [48] have described the activation of the AMP-activated protein kinase (AMPK) by metformin. AMPK is a key regulator of cellular energy balance, and activated AMPK switches cells from

☆ *Statement of funding:* Pauline Tartarin was supported by the "Ministère de l'Enseignement Supérieur et de la Recherche." This work was financially supported by Institut National de la Recherche Agronomique (INRA) and the national program "FERTiNERGY" funded by the French National Research Agency (ANR).

* Corresponding author. Fax: +33 2 47 42 77 78.
E-mail address: pascal.froment@tours.inra.fr (P. Froment).

http://dx.doi.org/10.1016/j.cryobiol.2014.02.006
0011-2240/

an anabolic to a catabolic state. Recent evidence suggests that AMPK is present in spermatozoa and this protein was involved in sperm physiology. Indeed, absence of this protein in mice altered spermatozoa motility, quality of the spermatozoa membrane and lowered the levels of antioxidant molecules [20,25,40]. Furthermore, AMPK has been shown to be activated in species with a natural freeze tolerance or during hibernation [19,35].

As metformin possesses a non-genomic action, it could be an interesting molecule to treat sperm, an apparently transcriptional inactive cell, and a molecule that could quickly modify spermatozoa metabolism in order to adapt cells to the surrounding environment. We have tested the effects of metformin on spermatozoa function and fertilizing capacity if added in cryopreservation media. To answer these questions we used standard sperm analysis tools and assessed the ability of mouse spermatozoa to fertilize oocytes during IVF.

Material and methods

Animals

Male Swiss strain mice and female C57BL/6 strain mice were housed under controlled photoperiod (lights on 05:00–17:00) with *ad libitum* access to food and water. Mice were purchased from Charles River (L'Arbresle, France). All animal procedures were carried out in accordance with the European legislation for animal experimentation (Directive 86/609/EEC) for animal experiments and with French legislation on animal research. The procedure reported in this paper was approved by the ethics committee of Val de Loire (CEEA VdL, Comité d'Ethique pour l'Expérimentation Animale du Val de Loire).

Chemicals and media

M2 Medium used for cumulus oocyte complex (COC) collection, mineral oil, raffinose and metformin was purchased from Sigma (Sigma Aldrich; Isle D'Abeau, France). Embryo culture was performed using Embryomax human tubal medium (HTF Medium, Millipore, St Quentin en Yvelines, France). All media and mineral oil used for culture were equilibrated for a minimum of 3 h before use at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Sperm collection

Twelve male mice 3–4 months of age underwent cervical dislocation, each animal's cauda epididymis was individually collected in saline and excess tissue and fat removed. The epididymis was incised in HTF medium and incubated for 5 min at 37 °C to allow the sperm to swim out and disperse. After incubation, spermatozoa were conserved in HTF. Spermatozoa from each male were mixed and split into two groups for the fresh and frozen treatments. Fresh spermatozoa were incubated at 37 °C for 30 min in 5% CO_2 in the absence or presence of either 50 μM, 500 μM or 5000 μM metformin. The experiment was repeated four times.

Sperm cryopreservation

The cryoprotectant agent (CPA) for male mice was composed of 22.5% raffinose (Sigma), 3% skim milk powder (Difco, Becton Dickson, Franklin Lakes, NJ) in HTF. The osmolarity of the cryopreservation medium was verified to be within 480 and 500 Osm/L as has previously been described [47] and filter sterilized using a 0.22 μM filter (Millipore, St. Quentin en Yvelines, France). During the cryopreservation process, spermatozoa were incubated for 30 min at 37 °C in CPA and in the absence or presence of either 50 μM, 500 μM or 5000 μM metformin as described above. Then, forty microliters of the spermatozoa/CPA/metformin mix was then cryopreserved using insemination straws (CryoBio System, Saint Ouen-sur-Iton, France) by exposure to liquid nitrogen vapor for 10 min and stored under liquid nitrogen until use. Insemination straws were placed 10–15 cm above the liquid nitrogen surface during the freezing process. Three insemination straws per treatment were used for subsequent analysis. Sperm was thawed after at least 2 weeks of cryopreservation in liquid nitrogen. Sperm were thawed for 10 s in a 37 °C water bath then incubated in HTF at 37 °C for 15 min in 5% CO_2 in a cell incubator before analysis.

Oocyte collection and in vitro fertilization

Sperm from Swiss mice were used as it has previously been shown that inbred C57BL/6 strain embryos are particularly susceptible to the stresses associated with embryo culture, whereas hybrid embryos are more resistant [24]. To collect mature metaphase II (MII) oocytes, 28 C57BL/6 strain adult mice in total were primed with 5 IU pregnant mare serum gonadotropin (PMSG) (Intervet, Boxmeer, Holland) by intraperitoneal injection followed 46 h by a 5 IU injection of human chorionic gonadotropin (hCG) (Intervet, Boxmeer, Holland). In warmed M2 medium, cumulus oocyte complexes (COCs) were recovered from the oviducal ampullae approximately 12 h after hCG stimulation. Following collection, COCs were washed three times in HTF medium. Approximately 30 COCs were randomly placed into a 4-well dishes containing 200 μL of HTF medium per well. Cryopreserved Swiss sperm was prepared for *in vitro* fertilization (IVF) by thawing straws in a 37 °C water bath for 10 s. The sperm were then directly expelled into the IVF dish containing the mature COCs. Following 5 h of co-incubation, presumptive zygotes were washed three times to remove cumulus cells and excess spermatozoa and placed into 20 μL drops of HTF medium under mineral oil. Embryo assessments were performed every 24 h for three days. The experiment was repeated 4 times, with a total of at least 130 oocytes inseminated per condition.

Analysis of plasma membrane integrity

Cell plasma membrane integrity was evaluated by a fluorescent staining method to assess murine spermatozoa viability. Spermatozoa were stained with the components of a live/dead spermatozoa viability kit (L-7011; Molecular Probes, Eugene, Oregon, USA) for 10 min at 37 °C in darkness. The final staining concentrations were 100 nM for SYBR-14 and 24 μM for propidium iodide (PI). Laser cytometric measurements were performed with an EPICS-XL flow cytometer (Beckman-Coulter, Villepinte, France). The fluorophores were excited by an air-cooled 15 mV argon ion laser and detected in two channel FL1 (530-nm band-pass filter) and FL2 (585-nm band-pass filter). Forward scattering and side scattering were recorded to define the size characteristics and provide gating parameters for selection of two fluorescent signals. After incubation, spermatozoa were analyzed in the flow cytometer and the results were expressed as the average percentage of control cells. A total of minimum of 4×10^4 gated events (based on the forward scatter and side scatter of the spermatozoa population recorded in the linear mode) were analyzed by using Win MDI 2.9 Software (Scripps Research Institute, USA).

Evaluation of sperm motility using Integrated Visual Optical System (IVOS)

Computer assisted sperm assessment (CASA) was performed with an HTM-IVOS (IVOS; Hamilton Thorne Biosciences, Beverly,

Please cite this article in press as: M.J. Bertoldo et al., Effect of metformin on the fertilizing ability of mouse spermatozoa, Cryobiology (2014), http://dx.doi.org/10.1016/j.cryobiol.2014.02.006