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Effect of genistein added to bull semen after thawing on pronuclear and sperm quality

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ABSTRACT

The aim of this research was to study the effect of different genistein treatments on bull sperm after thawing on pronuclear formation after *in vitro* fertilization (IVF) and on different sperm quality variables. Three experiments were performed. In Experiment 1, three treatments (Control, sperm incubation for 1 h at 37 °C with or without genistein) and two sperm concentrations during IVF (1 or 3×10^6 sperm/mL) were evaluated to study the influence of genistein on pronuclear formation (PNF). Sperm incubation for 1 h before IVF reduced PNF regardless of sperm concentration. However, after sperm incubation and with 3×10^6 sperm/mL in IVF, the genistein treatment group had greater fertilization rates than the untreated group. In Experiment 2, six treatments plus the control group were performed to study the effect of genistein (presence or not) and incubation conditions (30 min at 37 °C, 1 h at 27 °C or at 37 °C) on PNF using 3×10^6 sperm/mL for IVF. When incubation time was reduced to 30 min, PNF rate from the genistein treatment group was no different from either the control group or in the group in which incubation occurred for 1 h at 27 °C. In Experiment 3, the effect of several genistein treatments (control; genistein treatment for 30 min of incubation at 37 °C; genistein treatment for 1 h of incubation at 27 °C) on sperm motility, viability and DNA fragmentation were evaluated. Genistein did not improve sperm motility and, depending on the experimental group or time, it either reduced or had no effect on sperm motility. Genistein treatment did not improve sperm viability after 5 h of incubation. However, genistein treatment for 1 h at 27 °C decreased sperm DNA fragmentation compared with the control group after 5 h of sperm incubation. In conclusion, the treatment of bull sperm with genistein for 1 h at 27 °C could decrease sperm DNA fragmentation, although PNF rate after IVF and sperm motility were reduced.

1. Introduction

Application of reproductive technologies, such as cryopreservation or sex-sorting, may increase the oxidative stress status of sperm. For example, sperm washing and dilution in other media to prepare insemination doses may increase the reactive oxygen species (ROS; reviewed by Walczak-Jedrzejowska et al., 2013). The presence of ROS

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affects sperm motility, lipid peroxidation and DNA fragmentation (Lopes et al., 1998; Walczak-Jedrzejowska et al., 2013). The use of antioxidants can mitigate the harmful effect of ROS both in fresh sperm (Lopes et al., 1998) and sperm stored at low temperatures (cooled or cryopreserved; Cheema et al., 2009) or sex-sorted (Li et al., 2012). However, in another study there was a decrease in frozen/thawed ram sperm motility with use of the majority of antioxidants studied when these were added after thawing (Mata-Campuzano et al., 2012). The addition of antioxidants in the freezing extender before the sperm cryopreservation process has also been evaluated (Gadea et al., 2005; Bucak et al., 2009). However, more desirable results were obtained in pigs and humans when antioxidants were added after thawing (cited in Gadea et al., 2013). The beneficial effect of reduced glutathione in thawing medium was observed for fertilization variables, but not for the sperm motility in pigs and bulls (Gadea et al., 2004, 2008).

Genistein is an isoflavone, a plant compound, with different properties such as antioxidant activity (Sierens et al., 2002) that has a protective effect on DNA fragmentation and motility of human sperm (Sierens et al., 2002; Martinez-Soto et al., 2010). However, genistein did not affect sperm motility in cats and cattle (Pukazhenthi et al., 1998; Menzel et al., 2007). Additionally, genistein is also a phytoestrogen that binds at low concentrations with the oestrogen receptor (Bingham et al., 1998). Genistein may inhibit the acrosome reaction induced by the zona pellucida (ZP) (cats: Pukazhenthi et al., 1998; cattle: Menzel et al., 2007) and also in sperm-ZP binding (Menzel et al., 2007). It is, therefore, important to study both the effect of sperm antioxidant treatment on sperm variables and also the interaction with oocytes and further pronuclear formation (PNF).

Assessment is also important of the effect of sperm incubation time and temperature, with and without genistein treatment, on *in vitro* fertilization (IVF) efficacy. In humans, Jensen et al. (2004) observed that 2 h-sperm incubation increased PNF after IVF. However, Long et al. (2013) observed a detrimental effect of incubation time of mouse sperm heads after intracytoplasmic sperm injection (ICSI) as time of incubation increased. This decrease in fertilization could be prevented by increasing the sperm concentration.

The aim of the present research was to study the effects of genistein treatments of bull sperm after thawing on PNF after IVF and different sperm quality variables, utilizing different times and temperatures of treatment and IVF sperm concentrations.

2. Materials and methods

Unless otherwise stated, all chemical products were purchased from Sigma–Aldrich Quimica (Madrid, Spain).

2.1. In vitro oocyte maturation and fertilization

The *in vitro* oocyte maturation and fertilization processes were performed as in previous studies with some modifications (Cebrian-Serrano et al., 2013; Ahumada et al., 2013). Handling medium (HM199) was composed of Hepes M199 (M7528) supplemented with 7.4% heat-inactivated foetal bovine serum (FBS; 10108-157, GIBCO®, Invitrogen) and antibiotics. Maturation medium was composed of M199 (M4530) supplemented with 10% FBS and 10 ng/mL of epidermal growth factor. Cumulus–oocyte-complexes (COC) were collected by aspiration from 2 to 8 mm diameter follicles of cattle ovaries at a slaughterhouse and were washed three times in HM199. After aspiration, COC with several surrounding layers of cumulus cells and uniform cytoplasm were selected, washed three times in HM199, and matured *in vitro* for 22–24 h in maturation medium. COC were matured at 38.5 °C and 5% CO_2 in humidified air.

For each process, straws with 0.25 mL of frozen semen were thawed at 37 °C in a water bath for 1 min. Volumes of 200 μL of pooled semen were centrifuged for 10 min at 2.1×10^3 r.p.m. ($295 \times g$; Eppendorf Mini Spin centrifuge) through a Bovi Pure® gradient of 1 mL bottom layer medium, in 1.5 mL microcentrifuge tubes (Eppendorf). The sperm pellet was isolated and washed twice in 750 μL of fertilization medium by centrifugation at 2.4×10^3 r.p.m. ($386 \times g$) for 3 min; first without capacitation inducers, and then, in the second centrifugation, with capacitation inducers in the medium. Approximately 50 μL of semen pellet remained after the final centrifugation and was diluted with approximately 100 μL of fertilization medium. After *in vitro* oocyte maturation, COC were washed three times in Fert-Talp fertilization medium (Parrish et al., 1988) with 10 μg/mL of heparin (H9399), and co-incubated with the sperm in 30 μL drops for 18–20 h in 5% CO_2 at 38.5 °C under mineral oil (M8410).

2.2. Genistein treatment

Genistein (G6649) was dissolved in DMSO in a stock solution at 1 mM (Martinez-Soto et al., 2010). Genistein treatment consisted of incubation of sperm in medium with 10 μM Genistein (Sigma G-6649; Martinez-Soto et al., 2010), isolated from the light. For Experiments 1 and 2, the sperm pellet remaining after centrifugation through Bovi Pure® gradient was diluted in 750 μL of HM199 with genistein. After incubation in genistein medium, the sperm pellet was washed twice in 750 μL of fertilization medium, as described above.

2.3. Fertilization assessment

After the IVF, zygotes were stripped of surrounding cumulus cells with a hyaluronidase (H4272) treatment (1 mg/mL in HM199) using a stretched glass Pasteur pipette. Zygotes were subsequently fixed and stained in a solution of ethanol with Hoechst 33342 (25 μg/mL; B2261) for at least 30 min. After staining, embryos were examined under epifluorescence microscope at ×400 and ×600 magnification and classified into four groups (Cebrian-Serrano et al., 2013): (1) no fertilized oocytes (no PNF), (2) normal fertilization (oocytes with two pronuclei), (3) polyspermic fertilization (oocytes with more than two pronuclei) and (4) others (oocytes with other abnormal fertilization characteristics).

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