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Osteopontin improves sperm capacitation and *in vitro* fertilization efficiency in buffalo (*Bubalus bubalis*)

Lucia Boccia, Serena Di Francesco, Gianluca Neglia*, Marina De Blasi, Valentina Longobardi, Giuseppe Campanile, Bianca Gasparrini

Department of Veterinary Medicine and Animal Production, "Federico II" University, Naples, Italy

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ABSTRACT

The aim of this study was to evaluate the effect of osteopontin (OPN), an ubiquitous acid glycoprotein, on *in vitro* sperm capacitation and on *in vitro* embryo production (IVEP) efficiency in buffalo. In experiment 1, after swim-up separation the sperm were incubated in Tyrode albumin lactate pyruvate medium in the absence of capacitating agents (control), with the standard concentration of heparin (0.01 mM) and three different concentrations of OPN (0.1, 1, and 10 mcg/mL), both in the presence and absence of heparin, for 2 and 4 hours. Capacitation was assessed indirectly by estimating the percentage of acrosome-reacted sperm after incubation with lysophosphatidylcholine. In order to determine the effect of OPN, in the presence of heparin, on fertilization (Experiment 2) and *in vitro* embryo development (experiment 3), *in vitro*–matured buffalo oocytes were fertilized in the presence of 0, 0.1, 1, and 10 mcg/mL of OPN. After IVF, the presumptive zygotes were dezonated, fixed, stained, and then evaluated microscopically. At Days 5 and 7 of culture, the cleavage and blastocyst rates were evaluated, respectively. Two hours of treatment with OPN at the two higher concentrations (1 and 10 mcg/mL) promoted *in vitro* capacitation of buffalo sperm (experiment 1). A synergic action of OPN with heparin was also done for all OPN concentrations tested. At 4 hours incubation, all treatments, including heparin (20.4%), improved ($P < 0.01$) capacitation compared with the control (16.2%). Interestingly, the best results were reported in all groups treated with OPN + heparin (40.8%, 38.6%, and 33.8%, respectively; $P < 0.01$). The addition of OPN to the IVF medium had a positive influence on total penetration, synchronous pronuclei formation (experiment 2), and IVEP efficiency (experiment 3). In particular, the two lower concentrations of OPN (0.1 and 1 mcg/mL), compared with the control, gave higher synchronous pronuclei formation (73.5%, 75.0%, and 46.5%, respectively; $P < 0.01$) and cleavage rates (70.3%, 71.6%, and 59.3%, respectively; $P < 0.01$). Interestingly, the treatments also improved blastocyst yields (29.3%, 30.3%, and 19.4%, respectively; $P < 0.01$). In conclusion, these results indicate that adding OPN to the IVF system improves IVEP efficiency by enhancing *in vitro* sperm capacitation and blastocyst yields in buffalo.

1. Introduction

The water buffalo (*Bubalus bubalis*) is viewed as a producer of both energy and protein in developing countries [1,2]. To enhance its genetic value and transform the buffalo population in these countries, the best approach is to use new technologies for assisted reproduction [2,3]. Although the efficiency of *in vitro* embryo production (IVEP) has greatly increased in recent years in buffalo [4], the cleavage rate is still low compared with most other domestic species [4], and there is substantial evidence to

* Corresponding author. Tel.: +39 081 2536063; fax: +39 081 292981.
E-mail address: neglia@unina.it (G. Neglia).

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suggest that *in vitro* fertilization (IVF) is a critical step [4]. Many factors are known to affect IVF efficiency [4], such as sperm quality, the bull, the environment, the appropriate time of insemination, and appropriate capacitation of frozen-thawed sperm. Indeed, sperm need to undergo capacitation to acquire fertilizing ability. This process, which occurs *in vivo* within the female genital tract, must be induced *in vitro*. It is not possible to rule out that sperm capacitation is impaired in the buffalo IVF system currently used. Another limiting factor is the poor viability of *in vitro*–produced buffalo embryos due to suboptimal culture conditions [5].

Both fertilization and early embryo development *in vivo* occur in the oviduct and, despite many efforts to improve embryo culture systems in different species, this organ remains irreplaceable for appropriate embryo development. A practical approach to mimic the oviduct environment for optimizing IVEP efficiency is to identify the key molecules and ensure their subsequent incorporation in the *in vitro* system. One of the oviductal proteins that could be used *in vitro* to maximize the efficiency of IVF is osteopontin (OPN), which plays an important role in several species [6,7]. OPN, also known as phosphoprotein 1, is an acidic glycoprotein with a cell-binding domain comprising GRGDS, glycine-arginine-glycine-aspartate-serine [8,9], which is present in the female reproductive tract, namely in the oviduct, uterus, and placenta [10]. Gabler et al. [11] suggested that this protein, which belongs to a family of integrins, is synthesized in the bovine oviductal epithelium and released into oviductal fluid. Integrins are present on the surface of various cells and they promote cell adhesion [12]. Almeida et al. [13] showed that integrins can facilitate the process of fertilization in mammals by acting on sperm-oocyte binding, as they have been described on the surface of oocytes [13,14] and spermatozoa [15].

In cattle, several studies with the use of specific antibodies have shown that OPN is one of the seminal plasma proteins associated with fertility [16,17]. Furthermore, Henault et al. [18] found that exposure to accessory sex gland fluids of high-fertility bulls improves the sperm fertilizing ability of low-fertility bulls. Interestingly, Moura et al. [19] found that high-fertility Holstein bulls have greater concentrations of OPN in their accessory sex gland fluids than low-fertility bulls. OPN has also been detected in buffalo semen, at greater concentrations in the seminal plasma than in sperm cells [20], suggesting that OPN is produced by the ampullae and seminal vesicles, similar to what was reported for cattle [20]. Interestingly, buffalo semen frozen by standard procedures showed up to a 50% reduction in the amount of OPN [20].

All the above studies pointed toward testing the effect of OPN supplementation in the *in vitro* system to improve efficiency. Hao et al. [6] reported a positive effect of OPN, added to the IVF medium, on the acrosome sperm reaction and hence fertilization in swine. It was also reported that pretreatment of bovine oocytes and sperm with anti-OPN antibodies reduces the fertilization rates [7]. Moreover, it was found that OPN triggers *in vitro* capacitation of frozen-thawed sperm and improves post-fertilization embryo development in cattle [21].

Therefore, the aim of this study was to evaluate the effect of OPN on *in vitro* capacitation of frozen-thawed buffalo sperm for IVF (experiment 1). A further objective was to verify the effectiveness of OPN supplementation during IVF on fertilization (experiment 2) and overall IVEP efficiency (experiment 3).

2. Materials and methods

Unless otherwise stated, reagents were purchased from Sigma-Aldrich (Milan, Italy).

2.1. Assessment of sperm capacitation

In order to evaluate sperm capacitation (experiment 1), frozen spermatozoa from three Mediterranean buffalo bulls, previously tested for IVF, were thawed at 37 °C for 40 seconds, mixed to neutralize the bull effect, and separated by swim-up procedure in Sydney IVF Gamete Buffer (Cook, Nova Milanese, Italy, K-SIGB-100) for 1 hour to select only the motile population. Samples of semen were assessed immediately after thawing and after swim-up separation to evaluate the incidence of acrosomal loss in nontreated cells. Trypan blue (T8154) was used first to differentiate live spermatozoa from dead spermatozoa, and then the dried smears were fixed in 37% formaldehyde (F15587) and stained with Giemsa (GS500) for acrosome evaluation by microscopic examination [22]. The remaining spermatozoa were incubated in Tyrode albumin lactate pyruvate (TALP) medium in the absence of capacitating agents (control), with the standard concentration of heparin (0.01 mM), and with OPN (O3514) at three different concentrations (0.1, 1, and 10 mcg/mL), both in the presence and absence of heparin, for 2 and 4 hours. The range of concentrations of OPN was chosen after a preliminary dose response trial that showed a toxic effect at concentrations higher than 10 mcg/mL OPN.

At the end of incubation, capacitation was assessed indirectly by estimating the percentage of acrosome-reacted sperm (ARS) after 15 minutes incubation with lysophosphatidylcholine (L1381), a fusogenic agent known to induce acrosome reaction only in capacitated sperm [23]. To evaluate the sperm viability and the acrosome reaction, the sperm were fixed and stained with Trypan blue–Giemsa [22]. Sperm were counted under differential interference light microscopy and were classified as follows: acrosome-intact live, acrosome-intact dead, acrosome-reacted live, and acrosome-reacted dead. The experiment was repeated three times, and for each replicate, at least 200 sperm were evaluated per slide. In order to compare the efficiency of capacitation among groups, the percentages of ARS were calculated out of the total live cells.

2.2. In vitro embryo production

2.2.1. Oocyte collection and in vitro maturation

Buffalo ovaries were collected from a local abattoir and transported to the laboratory within 4 hours of slaughter at approximately 30 °C to 35 °C in physiological saline supplemented with 150 mg/L kanamycin (Gibco, Paisley, UK; 11815-024). Cumulus-oocyte complexes (COCs) were