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NON-ANIMAL MACROMOLECULES AS AN ALTERNATIVE TO BOVINE SERUM ALBUMIN IN THE BULL SPERM CAPACITATION MEDIUM: PRELIMINARY RESULTS

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Abstract

The aim of this paper was to compare two non-animal macromolecules (methyl cellulose and hydroxyethyl-starch) as an alternative to bovine serum albumin supplement in the bull sperm capacitation medium. Attention was paid to the rate of penetrated oocytes and to the polyspermic fertilization in order to assess the effect of these substances on bull semen capacitation.435 class one and two oocytes were matured in TCM -199supplemented with bovine serum albumin (3mg/ml) and FSH (0.88 mg/ml) for 24 hours at 38.5°C, 5% CO_2 in saturated humidity. 21 straws from one single bull ejaculate were used. Sperm motility was assessed using Sperm Vision 3.7® (Minitübe, Germany) before the separation process. Sperm preparation was done on a commercial density gradient medium (BoviPure®, Nidacon, Sweden). Three groups were formed: the bovine serum albumin group (BSA at 6mg/ml), the methyl cellulose group (MC at 0.1mg/ml) and the hydroxyethyl-starch group (HES at 10mg/ml). Matured oocytes were co-incubated for 18 hours with the sperm (38.5°C, 5% CO_2 in saturated humidity). After 18 hours, the oocytes were denuded, fixated and stained with aceto-orcein in order to assess the penetration and pronuclear formation.77.80%±14.82% of the oocytes in the BSA group were not-penetrated, 22.20%±14.82% were penetrated and3.37%±2.37% were with polyspermy. In the MC and HES group respectively, 71.18%±21.66% and 60.23% ± 23.04% of the oocytes were not-penetrated, 28.82%±21.66% and 39.77%±23.04% werepenetrated and8.71%±6.3% and 13.74%±8.87% were with polyspermy. The physical characteristics of methyl cellulose and hydroxyethyl-starch were considered suitable for the in vitro production; they are all white powder, colourless and odourless in aqueous solution. The results showed that, there are no significant differences ($p < 0.05$) between methyl cellulose and bovine serum albumin regarding the penetration rate and the polyspermic fertilization, thus making methyl cellulose suitable to be used as an alternative supplement to the capacitation medium for bull semen.

Key words*: bovine serum albumin, bull sperm, capacitation medium, non-animal macromolecules.*

INTRODUCTION

Bovine serum albumin (BSA) is extensively added to all the mediums for in vitro fertilization. Its role is to prevent cells from sticking to themselves or to the plastic ware and to provide nitrogenous substrate for cellular metabolism (Matson and Tardif, 2012). Additionally, the bovine serum albumin induces the efflux of cholesterol from the sperm membrane, the first crucial step in the sperm capacitation process (Naseer, 2014). In human medicine, animal or human blood-derived proteins are avoided in all the culture media for assisted reproductive technologies. This is based on the desire of excluding any risk of infection with hepatitis or Creutzfeldt-Jakob disease or with other viruses and infective agents that may appear in the process. Researchers in human medicine have considered plant-derived products such as enzymes for removing the cumulus cells (Parinaud and Vieitez, 1998) or culture medium supplements (Parinaud and Milhet, 1998; Parinaud and Milhet, 1999). Nevertheless, the cost of these plant-derived proteins must be

taken into consideration as it is substantially lower (a very important aspect for veterinary medicine and in vitro embryo production).
This study aims to investigate the efficiency of two different non-animal macromolecules as a supplement to capacitation medium for bull sperm cells and to compare these supplements efficacy to bovine serum albumin. In order to assess the performance of those non-animal macromolecules, the fertilization rate was taken into consideration (attention was paid to the rate of penetrated oocytes and to the polyspermic fertilization). Also, the physical characteristics of the supplements and their potential acceptability were studied.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

Collection and maturation of the oocytes

A total of 300 bovine ovaries were collected from adult cows in a local slaughterhouse and transported in NaCl 0.9% solution at 35°C to the laboratory. Only follicles between 2 and 8 mm diameter were aspired using 18G needles attached to a vacuum pump in 50 ml tubes. The sediment was taken out using a Pasteur pipette and transferred into a screened Petri dish (90 mm diameter) with modified PBS-Dulbecco with Ca^{2+} and Mg^{2+} (mPBS, Biochrom®, Berlin) supplemented with glucose (1 mg/ml), pyruvate (27.7 mg/ml), penicillin (0.02 mg/ml), streptomycin (0.04 mg/ml), heparin (0.01 mg/ml) and bovine serum albumin (0.3 mg/ml). The cumulus oocyte complexes (COCs) were classified as described by Leibfried and First (Leibfried and First, 1979) and onlyclass one and twoCOCs, with compact layers of cumulus cells and homogeneous cytoplasm, were selected and washed in mPBS. The selected COCs were washed twice with TCM-199 supplemented with bovine serum albumin (3 mg/ml), pH 7.4, then transferred to a 4-well dish (NUNC, Thermofisher, USA) containing 400µl TCM-199 supplemented with bovine serum albumin (3 mg/ml) and FSH (0.88 mg/ml), for washing. Finally, they were placed into the final 4-well dish with the same medium for maturation (24 hours at 38.5°C, 5% CO_2 and saturated humidity).

Sperm preparation

During the experiment 21 straws from a single bull ejaculate were used (0.25 ml straws, 15 x 10^6 sperm per straw). The experiment was repeated 7 times in 7 different days. Sperm motility was assessed before the sperm separation process using a Computer-Assisted-Sperm-Analysis system (Sperm Vision 3.7®, Minitübe, Germany) and the mean motility of the sperm was ranged at 60%.
Motile sperm was obtained using a commercial density gradient medium (BoviPure®, Nidacon, Sweden) and all the protocol was followed according to the producers recommendations. Briefly, for each sperm sample three Eppendorf tubes were prepared with 40%, 80% BoviPure and the wash one containing 1000µl BoviWash. These tubes were incubated for at least 30 minutes in 38°C. Afterwards, using a sterile pipette, 500µl BoviPure 40% were carefully transferred on top of the bottom layer of 500µl BoviPure 80%. The content of the thawed and cleaned sperm straw was slowly emptied onto the BoviPure gradient. The Eppendorf tube was centrifuged at 300 x g for 15 minutes at room temperature. After centrifugation, the supernatant was removed. The remaining pallets were transferred to the wash tube containing 1000µl of BoviWash solution and centrifuged again at 300 x g for 5 minutes. The supernatant was removed, leaving the pallet. The pallet was diluted using the capacitation medium to a final concentration of $1x10^6$sperm/ml in the IVF drop. After this procedure the sperm concentration was determined using an Improved Neubauer haemocytometer as described previously by Mahmoud et al. (1997).
Three groups were formed: the bovine serum albumin group (BSA), the methyl cellulose group (MC) and the hydroxyethyl starch group (HES).

In vitro fertilization

Modified Tyrode-Lactate medium (Bavister and Yanagimachi, 1977) containing heparin, gentamycin and hypotaurine-epinephrine was used for the *in vitro* fertilization and it will be further referred as modified TALP. The

capacitation/fertilization medium was supplemented, depending on the experimental group, with: 1) BSA (6 mg/ml) and sodium pyruvate (0.22 mg/ml); 2) MC (0.1 mg/ml) and sodium pyruvate (0.22 mg/ml); 3) HES (10 mg/ml) and sodium pyruvate (0.22 mg/ml). After maturation, the recovered COCs were washed in 200µl drops of modified TALP and then transferred into the 60µl drops of the same medium for the fertilization (Haenisch Woehl, 2003).
Fertilization was carried out at 38.5°C under 5% CO_2 in 100% humidified air for 18 hours.

Assessment of sperm penetration of oocytes *in vitro*

After 18 hours of co-incubation, the fertilized oocytes were denuded in 400µl modified D-PBS by repeated pipetting and then washed three times.
The denuded oocytes were mounted, fixed for 48 hours at 4°C in ethanol-glacial acetic acid (3:1), stained with 1% (v/v) orcein in 45% (v/v) acetic acid and examined under phase-contrast microscopy at a magnification of 200 or 400x (Nikon Eclipse E400) in order to assessthe sperm penetration of oocytes.
Oocytes that had been penetrated were identified when an enlarged sperm head or male pronucleus with its accompanying sperm tail was present in the oocytes. Oocytes with more than two pronuclei and a clear second polar body, but without a sperm tail, were also considered penetrated (polyspermy).

Statistical analysis

Values for penetrated oocytes, not-penetrated oocytes and polyspermy were evaluated using analysis of variance (One Way ANOVA) in the IBM® SPSS® Statistics Version 21 software program.

RESULTS AND DISCUSSIONS

The values of penetrated oocytes, not-penetrated oocytes and penetrated oocytes with polyspermy (mean ± standard deviation) for the three groups are given in Table 1 and Figure 1 and 2.

Table 1: Comparative values of penetrated oocytes,not-penetrated oocytes and polyspermic fertilization (mean ± standard deviation)

Capacitation medium supplement	Penetrated oocytes	Not-penetrated oocytes	Polyspermy
BSA	22.20 ± 14.82	77.80 ± 14.82	3.37 ± 2.37
MC	28.82 ± 21.66	71.18 ± 21.66	8.71 ± 6.3
HES	39.77 ± 23.04	60.23 ± 23.04	13.74 ± 8.87

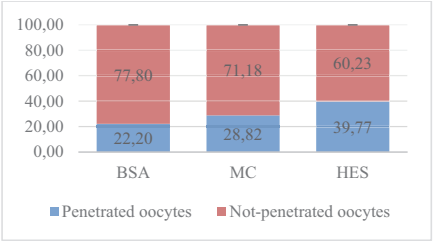


Figure 1. Comparative values of penetrated oocytes and not-penetrated oocytes

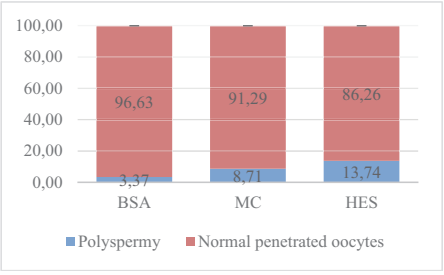


Figure 2. Comparative values of polyspermic fertilization and penetrated oocytes with no polyspermy

The penetrated oocytes (Figure 3) did not differ significantly ($p < 0.05$) between the methyl cellulose (37/150, 28.82 ± 21.66%) and thehydroxyethyl-starch group (48/134, 39.77 ± 23.04%) compared with the bovine serum albumin group (34/149, 22.20 ± 14.82%) as showed in Table 2.
The not-penetrated oocytes (Figure 3) did not differ significantly ($p < 0.05$) in the methyl cellulose group (113/150, 71.18 ± 21.66%) and in the hydroxyethyl-starch group (86/134, 60.23 ± 23.04%) compared with the bovine serum albumin group (115/149, 77.80 ± 14.82%) as showed in Table 2.

Table 2: Effect of the capacitation medium supplement on the penetrated / not-penetrated oocytes

Capacitation medium supplement		Mean difference
BSA	MC	-,06616
	HES	-,17573
MC	BSA	,06616
	HES	-,10956
HES	BSA	,17573
	MC	,10956

*The mean difference is significant at the 0.05 level

The polyspermic fertilization (Figure 3) was significantly higher ($p<0.05$) in the hydroxyethyl-starch group (15/134, 13.74%± 8.87%) compared with the bovine serum albumin group (5/149, 3.37 ± 2.37%), but it did not differ significantly ($p < 0.05$) between the methyl cellulose group (12/150, 8.71 ± 6.3%)and the bovine serum albumin group (5/149, 3.37 ± 2.37%) as showed in Table 3.

Table 3: Effect of the capacitation medium supplement on the polyspermic penetration of the oocytes

Capacitation medium supplement		Mean difference
BSA	MC	-,05338
	HES	-,10373*
MC	BSA	,05338
	HES	,05035
HES	BSA	,10373*
	MC	,05035

*The mean difference is significant at the 0.05 level

The results of the present study show that there are non-animal macromolecules than can substitute bovine serum albumin from the capacitation medium without damaging the sperm cells and the oocytes. Unfortunately, when referring to the rate of polyspermy, only methyl cellulose is considered to give the same results as bovine serum albumin.

Attempts have been made in the past in order to substitute BSA with other non-animal proteins in several culture media. One of these attempts was made by Biggers and his collaborators (Biggers and Summers, 1997) who have investigated the effect of replacing BSA with polyvinyl alcohol (PVA) and/or amino acids on mouse zygote development. They concluded that PVA could not substitute completely BSA in the mouse embryo culture medium. They followed the blastocyst rate during their experiments and observed that blastocyst development was only slightly less than with BSA, but the rate of partial hatching was significantly less. Substitution of BSA with PVA lowered the overall response but did not lead to major perturbation.

The present study was focused on the penetration of the oocytes in order to avoid the oocyte influence on the fertilization and assess only the ability of BSAs replacements to induce sperm capacitation. The substances used in this study in order to substitute BSA from the capacitation medium were used in human medicine for short term culture of human sperm at the same concentrations (Matson and Tardif, 2012) giving encouraging results and only the best of them were chosen for bull sperm capacitation.

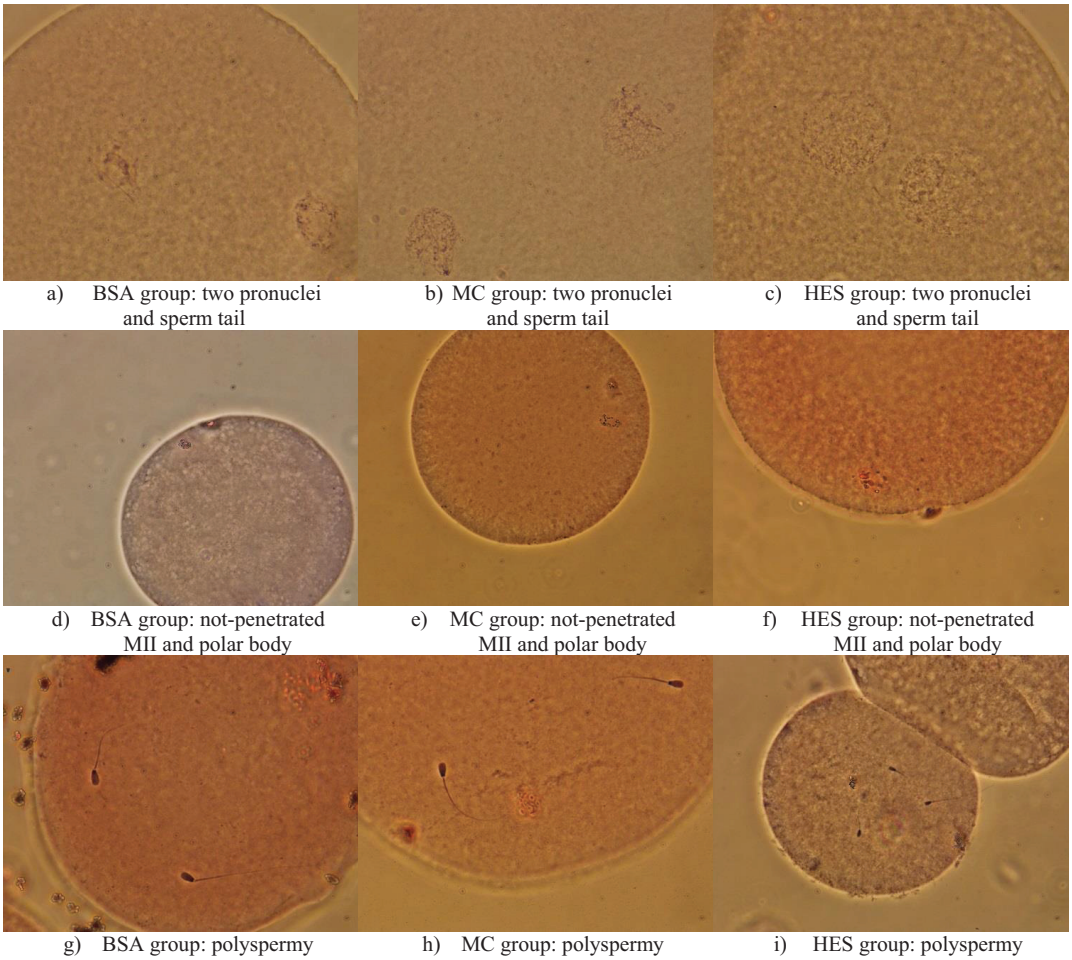
The physical characteristics of methyl cellulose and hydroxyethyl-starch were considered suitable for clinical use; they are all white powder, colourless and odourless in aqueous solution.

Matson and Tardif concluded in their study that hydroxyethyl-starch had poor solubility and had to be centrifuged resulting in a saturated solution of undefined concentration (Matson and Tardif, 2012). In the present study, this problem had been solved by sterile filtration (0.2µm, 5.7cm^2 filter) of the medium containing these supplements before their use (Insufil, Fresenius Kabi, Germany). This poor solubility aspect was noticed only regarding hydroxyethyl-starch.

Additionally, both methyl cellulose and hydroxyethyl-starch did not significantly modify the mediums pH (initially 7.8) after 3hours of incubation before being used for the fertilization (BSA – pH 7.93, MC – 7.96 and HES – 8.03).

Protein-free medium have been used before but only in human assisted reproductive technologies. SMART1® medium (Parinaud, Milhet et al. 1998) was developed in order to support fertilization and it was shown to give good fertilization rates and hence supported capacitation and avoided premature acrosome reaction. Another series of media, the ART-7 series (Ali and Shahata, 2000) ensured a sperm survival rate of approximately 80% and has been shown to support both fertilization and embryo development. It contained undefined supplements, but these were likely to include methyl cellulose (Ali, 2009).

There are no data available at this moment for protein-free medium designed for assisted reproductive technologies in veterinary medicine, so that our results cannot be compared with other studies results. These preliminary results will be completed with more than one bull ejaculate in order to avoid the ejaculate effect and individual influence on the results.



a) BSA group: two pronuclei and sperm tail
b) MC group: two pronuclei and sperm tail
c) HES group: two pronuclei and sperm tail
d) BSA group: not-penetrated MII and polar body
e) MC group: not-penetrated MII and polar body
f) HES group: not-penetrated MII and polar body
g) BSA group: polyspermy
h) MC group: polyspermy
i) HES group: polyspermy

Figure 3. Morphological analysis of the oocytes. Oocytes were stained with aceto-orcein dye to analyse chromatin configuration: penetrated oocytes (the presence of both male and female pronucleus and the sperm tail), not-penetrated oocytes (MII stage and the presence of the polar body) and polyspermic fertilization (the presence of more than one sperm inside of the oocyte or more than two pronuclei)

CONCLUSIONS

In conclusion, no significant differences were observed between methyl cellulose and bovine serum albumin regarding the penetrated oocytes and the polyspermic fertilization. Thus MC is suitable to be used as a supplement in the capacitation medium for bull sperm.

The present study showed no significant differences between hydroxyethyl-starch and bovine serum albumin regarding the penetrated oocytes, but found a significant difference when the polyspermic fertilization was taken into consideration, thus suggesting that hydroxyethyl-starch may have a negative effect on the penetration of the oocytes.

Further studies are needed in order to assess the real efficiency of these non-animal macromolecules on the embryo development when added as supplements in bull sperm capacitation medium.

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