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A New Boar Sperm Dilution Medium for Artificial Insemination Technology

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Abstract

For artificial insemination, the sperm is diluted with different media in order to obtain a higher volume of sperm and to maintain an increased survival rate of spermatozoa in vitro.

This paper looked into the effects of glycosaminoglycans (chondroitin sulphate and hyaluronic acid) and proteoglycans (obtained from swine follicular fluid) added to a dilution medium for boar sperm (BTS) on the functional parameters of spermatozoa.

Our results indicate that the highest percentage of living boar spermatozoa corresponds to sperm samples diluted with BTS medium containing 0.05% chondroitin sulphate. Moreover, the addition of this glycosaminoglycan to the dilution medium maintains the normal morphology of spermatozoa and promotes their acrosomal reaction.

In conclusion, the new dilution medium obtained by us may be used in the field of swine artificial insemination.

Keywords: boar sperm, glycosaminoglycans, proteoglycans, dilution medium, artificial insemination

Introduction

The artificial insemination technology consists of collecting, diluting and introducing the sperm (by the means of special instruments) into female genital tract without mating. In view of artificial insemination, the semen is diluted before use. The dilution must be done so as to obtain a higher volume of sperm and to maintain an increased survival rate of spermatozoa *in vitro*. The dilution media used in practice have to be chosen so that they allow a high dilution extent for an efficient use of the semen. At the same time, the dilution media have to maintain the viability and fertilizing ability of spermatozoa following their incubation in the mentioned media.

In the zootechnical practice, the synthetic dilution media (with accurately dosed constituents) for boar sperm were proved to be the best. For instance, at the unit for artificial insemination *S.C. Romsuintest-Peris S.A.,* the raw boar sperm is diluted with BTS medium

(Beltvulle Thawning Solution, L.A. Johnson et al. formula, Minitüb, Germany), which contains substances that provide spermatozoa protection against toxic agents, substances for the medium isotonicity as well as substances with antimicrobial effect [1].

Viability, motility and *in vitro* inducing of the acrosomal reaction are key functional parameters for spermatozoa. Their normal values are a prerequisite for spermatozoa from diluted samples used in artificial insemination.

Previous studies performed abroad [2] as well as some results of our own research [3] demonstrated that some glycosaminoglycans or proteoglycans have the ability to maintain *in vitro* spermatozoa viability and motility and to induce the acrosomal reaction.

Proteoglycans are complex macromolecules composed of a protein core to which one or more sulphated glycosaminoglycan side chains are attached [4-6]. Glycosaminoglycans are polyanionic chains of variable length constructed from disaccharide repeating units that contain a hexosamine residue [7,8]. In mammalian tissues there are four major classes of glycosaminoglycans: hyaluronic acid, the chondroitin sulphates, keratan sulphate and the heparan sulphate – heparin class [9,10].

Spermatozoa are in contact with some proteoglycan/glycosaminoglycan (PG/GAG) types when passing through the female reproductive tract towards the insemination site. Thus, our aim was to study the effect of these macromolecules added to the formula of a dilution medium on the functional parameters of boar spermatozoa.

Materials and Methods

I. ***Biological material***. The raw sperm was collected from three different boar breeds: the Great White, Landrace and Peris 345 Synthetic Line. The sperm samples were collected by means of an artificial vagina, under sterile conditions and transported under isothermal conditions to the laboratory.

II. ***Dilution media used***. In order to dilute the boar raw sperm, the following different media were used:

- modified Tyrode medium, containing 0.95% NaCl, 0.05% $CaCl_2$ and 0.05% of the following PG/GAG:

a) chondroitin sulphate (CS) extracted from bovine trachea [11];
b) hyaluronic acid (HA) from bovine umbilical cord [12];
c) proteoglycans from swine follicular fluid [13].

- BTS medium containing 0.05% PG/GAG (a-c);
- Tyrode medium (as control);
- BTS medium (as control).

III. ***Raw sperm processing with the dilution media***. Sperm samples of known features were mixed at a ratio of 1:3 (v/v) with dilution media mentioned at point II. After dilution, the samples were gently stirred until complete homogenization. The diluted samples were maintained in 5% CO_2 atmosphere, for 24, 48 and 72 hours, at 37ºC.

IV. **Methods to evaluate the functional status of spermatozoa after dilution**.

- *The motility of spermatozoa* from the diluted samples was estimated after their examination onto a glass slide covered with a coverslip.

- *The viability of spermatozoa*. A volume of diluted sperm is mixed with an equal volume of 1% Trypan Blue in 0.1M phosphate buffer (pH 7.5) and maintained for 10 min, at room temperature. The mixture was smeared on microscopic slides and examined by light microscopy with a submersion objective. In the microscopic field, living spermatozoa (colourless) and dead spermatozoa (dark blue coloured) were observed.
- *Morphological features of spermatozoa after the dilution and the evaluation of their ability to undergo* in vitro *acrosomal reaction.* Sperm were fixed with 4% paraformaldehyde solution (110mM Na_2PO_4, 2.5mM NaH_2PO_4, 4% paraformaldehyde pH 7.4) for 10 min, at 24ºC. Sperm were centrifuged and washed twice using 1.5 mL of 100mM ammonium acetate (pH 9.0). The final sperm pellet was resuspended in 1 mL of 100 mM ammonium acetate and 50 μl of the sperm suspension was smeared on glass microscope slides and air dried. Sperm on the slides were incubated in freshly made Coomassie stain (0.22% Coomassie Blue G-250, 5% methanol, 10% glacial acetic acid, 40% water) for 2 min. Slides were washed thoroughly using distilled water to remove excess stain. Slides were air-dried and coverslips were placed on slides and sealed using a mounting medium (Canada balm). A minimum of 10 fields per slide (> 100 sperm) and a total of 6 slides per experiment were observed.

Results and Discussions

Parameters of raw sperm samples

After collection, the raw sperm samples were analysed from the point of view of the spermatozoa density and motility. The results are shown in **Table 1**.

Table 1. Features of boar raw sperm samples

Great White breed			**Landrace breed**			**Peris 345– LS breed**		
Boar code	Density (mill)	Mobility (%)	Boar code	Density (mill)	Mobility (%)	Boar code	Density (mill)	Mobility (%)
A1	544	73	B1	367	65	C1	450	73
A2	523	70	B2	477	67	C2	450	70
A3	502	70	B3	521	74	C3	250	65
A4	583	71	B4	563	67	C4	300	60
A5	539	68	B5	534	74	C5	350	72
A6	468	72	B6	301	61	C6	300	60
A7	457	69	B7	479	72	C7	350	69

These raw sperm samples were then diluted with BTS medium (at a 1:3 ratio, v/v) and their motility and viability were tested after 24 h. The data shown in **Table 2** emphasized that the best results (70% motility; 65% viability) were obtained from the diluted sperm of A4 boar, Great White breed.

Therefore, further tests concerning viability, motility and ability of spermatozoa from diluted samples to undergo *in vitro* acrosomal reaction were made only on the samples obtained from A4 boar.

Table 2 – Features of diluted sperm after 24 hours

Great White breed			**Landrace breed**			**Peris 345 – LS breed**		
Boar code	Motility (%)	Viability (%)	Boar code	Motility (%)	Viability (%)	Boar code	Motility (%)	Viability (%)
A1	70	60	B1	63	55	C1	70	60
A2	68	60	B2	65	55	C2	65	55
A3	65	60	B3	70	60	C3	60	50
A4	70	65	B4	63	55	C4	58	50
A5	65	60	B5	70	60	C5	70	60
A6	70	60	B6	60	50	C6	58	50
A7	65	55	B7	70	60	C7	65	45

Analysis of sperm samples after dilution and sorting the best medium

Sperm samples with determined features were diluted in the media presented at point II of Materials and methods, at a ratio of 1 : 3 (v/v). After dilution, the samples were maintained in 5% CO_2 atmosphere, for 24, 48 and 72 hrs, at 37°C. After each incubation period, the samples were stained with 1% Trypan blue in phosphate buffer, pH 7.5 and alive spermatozoa were counted at a Nikon light microscope. It is known that Trypan blue is the stain commonly used to examine cell viability. Trypan blue does not penetrate living cells (colourless), but passes across the disorganized membrane of the dead cells (blue coloured).

The results obtained from these experiments are given in **Table 3**.

Table 3 – Spermatozoa viability after incubation in different dilution media for 24, 36 and 72 hrs.

Dilution media		**Spermatozoa viability (% from total spermatozoa)**		
		24 hrs	36 hrs	72 hrs
1.	Tyrode medium (control)	48	32	24
2.	Tyrode medium – CS	55	40	28
3.	Tyrode medium – HA	50	35	25
4.	Tyrode medium – PG	50	35	25
5.	BTS medium (control)	55	50	45
6.	BTS medium – PG	57	51	40
7.	BTS medium – CS	65	60	55
8.	BTS medium – HA	58	50	35

It can be noticed, from these results, that the highest percentage of alive spermatozoa corresponding to sperm samples diluted with BTS medium containing glycosaminoglycans or proteoglycans. Among these, the sample with the higher number of living spermatozoa after 72 hrs was that diluted in BTS medium with 0.05% chondroitin suphfate. This result is consistent with our previous observation that chondroitin sulphate is also efficient for *in vitro* inducing of acrosomal reaction in boar spermatozoa [14]. Thus, boar spermatozoa samples were incubated for 9 hrs, at 37°C, in physiological serum, modified Tyrode medium, and

Tyrode medium with 50 μg/ml chondroitin sulphate, respectively. After naphtol yellow and erythrosine B staining (to quantify the acrosomal reaction), it was noticed that in the presence of chondroitin sulphate a higher number of spermatozoa (58%) underwent the acrosomal reaction compared to control sample (42%).

Morphological features of spermatozoa after dilution in the selected medium

Morphological evaluation of spermatozoa refers to their structural integrity (presence of all its pieces : head, midpiece, tail) and the maintainance of their species' specific shape. The aim of the performed tests was to verify morphological integrity of boar spermatozoa diluted in BTS medium containing chondroitin sulphate after 72 hrs.

Acrosomal status determination is another parameter for assessing viable sperm (i.e. their fertilizing ability) after a period of *in vitro* preservation [15].

The acrosome reaction involves the fusion and vesiculation of the apical portion of the sperm plasma membrane with the outer acrosomal membrane at many sites on the sperm head inducing membrane vesiculation [16]. Disruption of these vesicles leads to the subsequent exposure of the acrosomal proteases (acrosine, hyaluronidase, etc.) to the extracellular environment. These enzymes have active roles in mediating sperm passage through the pellucide area surrounding the oocyte, thus serving the insemination process [17].

In this context, we have used the Coomassie Brilliant Blue G-250 staining method [18] to assess the spermatozoa morphology and ability to undergo the acrosomal reaction after their preservation in the selected dilution medium, for 72 hrs.

The light microscopic procedure provided an accurately observation tool for the studied cells' morphology. The intact acrosome and acrosome-reacted spermatozoa were easily and reliably identified as they were stained intensely blue in colour and lacked Coomassie staining over the acrosomal region, respectively.

These findings prove that the acrosomal status of spermatozoa is discernible using this simple staining method.

The spermatozoa samples diluted in media with or without chondroitin sulphate were examined by light microscopy. It was found that the studied cells have not modified their morphology (practically, no structural sperm aberrations were observed) (**Figure 1 A, B**).

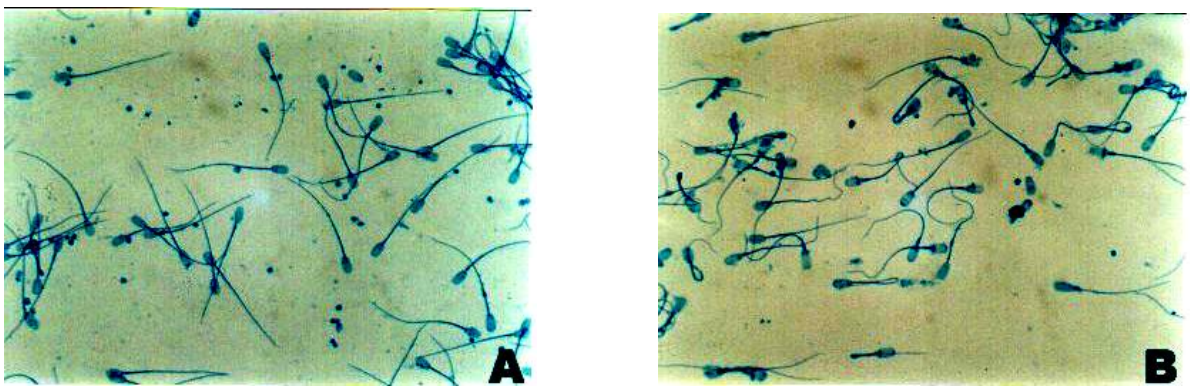

Figure 1 – Light micrograph showing the morphological integrity of spermatozoa after incubation at 18°C, for 72 hrs in: (**A)** – BTS medium (control); (**B)** – BTS medium containing CS

According to the literature, a recent method of estimating male fertility is based on assessing the ability of spermatozoa to undergo the acrosome reaction. The use of glycosaminoglycans to induce acrosomal reaction *in vitro* is a potentially valuable method for evaluating fertility [19, 20].

For these reasons, we assumed that chondroitin sulphate addition to the dilution medium of the seminal samples affects sperm function including an increased life span of *in vitro* spermatozoa and also inducing acrosomal reaction during incubation.

After the smears' staining by Coomassie Brilliant Blue method, it could be clearly observed the appearance of a greater number of acrosome-reacted spermatozoa in the dilution medium containing chondroitin sulphate compared to controls (spermatozoa diluted in BTS medium) (**Figure 2 A, B**).

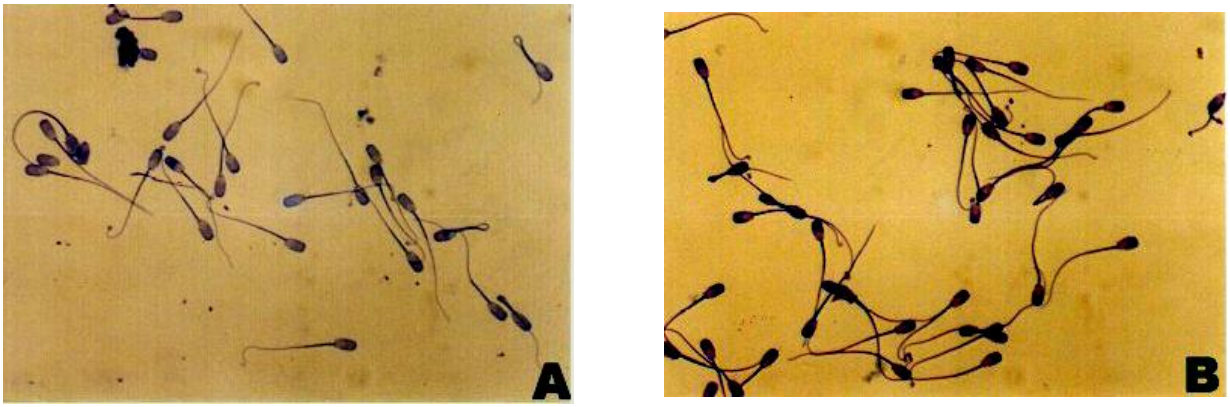

Figure 2 - Light micrograph showing the appearance of a greater number of acrosome-reacted spermatozoa (lack of blue staining in the head region) in the dilution medium containing CS (**A**), compared to the control diluted in BTS medium (**B**). Coomassie Brilliant Blue G-250 staining.

Conclusions

We propose a new dilution medium constituted of BTS medium and 0.05% CS which maintains *in vitro* viability of boar spermatozoa for 72 hours, at 37°C.

The addition of the glycosaminoglycan chondroitin sulphate to the dilution medium of boar seminal material maintains the normal morphology of spermatozoa and promoted their acrosomal reaction.

The dilution media with best results for preserving *in vitro* spermatozoa viability were provided to The Deprtment for the Research of Swine Breeding Periş. They were tested on several boar samples proceeded from different breeds and the results were consistent with our findings. Hence, our dilution medium may be used in the field of swine artificial insemination.

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