



Short communication

Reproductive efficiency of a new modified boar semen extender for liquid storage



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ABSTRACT

In the present study the Authors developed a new modified boar semen extender for short-term liquid storage, based on the use of amikacin sulphate and fructose rather than gentamicin and glucose. The new extender (ME-S) was evaluated and compared in vitro to commercial ones (CRONOSTM, TRIXcellTM) and to a modified extender designated for long term storage (ME-L) for progressive motility. Progressive motility was not different ($P > 0.05$) among extenders until 120 h of storage, as differences among extenders became significant ($P < 0.05$) at 144 and 166 h. Motility data across time were better for ME-S than TRIXcellTM ($P < 0.05$). No differences were observed about the morphology and membrane integrity (ORT) among the new extender (ME-S) and the commercial ones. Following the results of the in vitro comparison, an artificial insemination field trial was performed for reproductive efficacy. In this trial ME-L was not used because it was not completely reliable yet. A total of 1011 sows were bred: 506 with ME-S and 505 with a commercial one (CRONOSTM). The pregnancy rate for ME-S was 93.68% (474 pregnant sows), as the commercial extender resulted in 452 pregnancies (89.5%). The statistical comparison was significant ($P < 0.05$) and the number of live piglets born showed an increase of 52.

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1. Introduction

Over the last 25 years artificial insemination in pigs has developed enormously, both as a means for increasing animal production as well for improving genetic quality in herds. During this time the number of sperm per artificial insemination (AI) dosage has steadily decreased from about 6×10^9 to less than 3×10^9 , and this reduction of sperm numbers in the inseminate dose has greatly increased the efficiency of A.I. At the same time the shelf life of extended semen has become increasingly important for more flexible use of AI (Waberski et al., 2008). Swine sperm are very sensitive to the cooling, likely because of

the low cholesterol/phospholipids ratio of their membrane (Dubè et al., 2004); so frozen semen accounts for less than 1% of all inseminations in swine industry (Johnson et al., 2000). Moreover slowed genetics gains, lowered fertility and additional costs may limit use of frozen-thawed semen (Knox, 2011). This condition leads to use mostly fresh semen diluted in short-term extenders (Vyt et al., 2004); thus in swine artificial insemination a major challenge is the maintenance of the viability of the sperm for several days in the extender (Dubè et al., 2004). Fertility of the diluted semen doses usually decline as their preservation period increases and the viability of preserved boar semen is strongly influenced by the composition of the extender used. To meet the different needs of the swine industry, several extenders are commercially available for short and long-term liquid storage. The function of the extender is to supply the nutrients needed

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for the metabolic maintenance of the sperm cells, to control pH, osmotic pressure of the medium and inhibit microbial growth. The source of energy most commonly used in semen diluents is glucose, although other sugars (galactose, fructose, ribose or trehalose) have been tested generally yielding worse results (Gadea, 2003). Sperm morphology gives an indication of sperm viability (Britt et al., 1999), while sperm motility in a medium is an indicator of an active metabolism and the integrity of membranes (Johnson et al., 2000) and it is considered essential for fertilizing ability (Ivanova and Mollova, 1993; Vyt et al., 2004). Sperm motility and viability are fundamental factors to the success of reproduction (Dubè et al., 2004). Motility can be assessed subjectively by visual scoring (Dimitrov et al., 2007; Tardif et al., 1999), as in most AI centres, and objectively by Computer Assisted Semen Analysis (CASA) systems, that provide accurate information (López et al., 2009) and some motion parameters related to capacitation changes and fertility (García-Herreros et al., 2005; Vyt et al., 2008). The temperature during semen processing and storing, promotes the growth of most Gram negative bacteria (including *Escherichia coli* and some *Salmonella* and *Pseudomonas* species). Therefore, insemination with high contaminated sperm doses can interfere with fertilization resulting in high numbers of sows returning to oestrus (Kuster and Althouse 1997). Thus adding an antibiotic at the appropriate concentration improves sperm survival and, in turn, improves fertility results (Gadea, 2003). The present study was aimed at investigating: the in vitro immediate and progressive motility of boar semen diluted for liquid storage with different formulations of a modified extender; the reproductive efficiency of the chosen extender for best motility results on increasing pregnancy rate in sows; the effects on number of piglets born alive.

2. Materials and methods

The study was carried out in accordance with the Italian Legislation on animal care (DL 116/92).

Two different extender formulations for swine, were evaluated, named ME-S and ME-L (Table 1), during 12 trials. The semen was collected and processed from 15 boars of different breeds: seven mature crossbred Landrace × Large White, three hybrids (C21 and Goland), one Pietrain, 4 Duroc, ageing from 10 months to 2 years (average age 17 months). Nine of the semen donors were from a specialized centre in boar semen production, and six were from a farm practicing integral pig production; in both farms the animals were housed in individual pens and managed under similar conditions. All the boars were of proved fertility, and they were normally employed in commercial production of semen for AI. Before semen collections the animals were properly cleaned. Their ejaculates were collected by farms personnel using gloved-hand technique with a dummy, wearing non-spermicidal gloves (nitrile). The semen was collected in a pre-warmed (38 °C) plastic container with 500 ml capacity, without insulated cover cup, with a disposable filter stretched across the opening to separate out the gel component. The presperm fraction and sperm poor fraction were not collected and only the sperm rich fraction

Table 1
Composition (% w/w) of the two modified extenders used in the study.

Component	ME-S ^a	ME-L ^b
Fructose	78.60	27.22
Potassium chloride	1.56	–
Sodium bicarbonate	2.67	4.47
Na EDTA	2.67	6.06
Sodium citrate	12.00	30.30
TRIS	–	16.72
Citric acid	–	11.94
Amikacin sulphate	2.50	3.29

Fructose (Acef, Italy), potassium chloride, sodium bicarbonate, disodium ethylenediaminetetraacetate (NaEDTA), tri-sodium citrate dehydrate, citric acid, (Carlo Erba Reagents, Italy), tris-8hydroxymethyl aminometane (TRIS) (DBH Prolabo, Germany), amikacin sulphate (Sigma-Aldrich, USA).

^a Modified extender Short-Term.

^b Modified extender Long-Term.

was processed. For each trial both the extenders were used and compared with two commercial extenders (CRONOS™ by Medinova, Reggio Emilia, Italy and TRIXcell™ by IMV technologies, L'Aigle Cedex, France). Each trial was conducted with the following method: in the morning of each experimental session at the lab of Reproduction Unit in Parma the four extenders were prepared, by dissolving in 100 ml of purified water the powder formulations by means of a warmed magnetic stirrer, and maintained at 35 °C during the transport to the breeding centre until the semen collection. Immediately after collection each ejaculate was evaluated for colour (clear, cloudy, presence of blood, turbid or other uncommon colour), smell (typical, no smell, urine-smell, other), and weight/volume (g/ml). Sperm concentration was calculated by photometric means, using a spectrophotometer (Jenway LTD 60-51[®], Bibby Scientific Equipment Division, Staffordshire, UK). A drop (10 µl) of undiluted semen was examined on a warmed (37 °C) microscope slide overlaid with a coverslip and observed for sperm motility by phase contrast microscopy at 200 × magnification in at least four fields on the slide taking the average of these readings to obtain the final motility estimate. The semen was then diluted in each extender at 30 × 10⁶ spermatozoa/ml proportion to achieve a final concentration of 1.5 × 10⁹ spermatozoa in a total volume of 50 ml. Within the next 40 min, the diluted samples were transported to laboratory in a thermostat at 23–24 °C, then allowed to cool down at room temperature for 1 h and finally stored at 16 °C. Each sample was investigated in the following days (at 24, 48, 72, 96, 120, 144 and 166 h of storage) for progressive motility and clumping phenomena using a light microscope at 100 × magnification, until motility decreased below 40%. The progressive motility was determined as described for raw semen. The controls were always done by the same trained technician. Morphology was assessed using eosin–nigrosin staining following standard procedures (Shipley, 1999) and Osmotic Resistance Test (ORT) was performed according to the Martin Rillo et al. (1996) technique at 24, 48, 72, 96, 120, 144 and 166 h of storage. An aliquot of crude and diluted semen was referred to the laboratory of the Unit of Infectious Diseases of Animals (Parma University) and investigated for routine microbiology testing and antimicrobial susceptibility tests. Bacteriological swabs were obtained on raw and

Table 2

Least squares means (\pm SE) of extender \times time interaction on boar sperm motility (%).

Extender Time (hours)	Sperm motility (%)			
	ME-L ^a	ME-S ^b	CRONOS TM ^c	TRIXcell TM ^d
24	78.0 \pm 0.9	75.0 \pm 3.1	78.5 \pm 2.5	76.7 \pm 5.4
48	75.0 \pm 2.7	70.0 \pm 5.2	76.0 \pm 1.9	69.2 \pm 7.2
72	72.5 \pm 3.2	69.2 \pm 5.9	69.0 \pm 4.9	65.8 \pm 9.3
96	68.0 \pm 4.6	65.0 \pm 7.3	60.5 \pm 4.5	61.2 \pm 9.4
120	63.0 \pm 8.3	59.2 \pm 9.1	55.0 \pm 5.3	55.8 \pm 9.3
144	67.4 \pm 1.3 c	54.2 \pm 10.0 b	53.0 \pm 6.2 ab	41.5 \pm 12.3 a
166	62.4 \pm 1.6 c	56.6 \pm 3.7 bc	39.0 \pm 12.1 a	43.4 \pm 11.8 ab
Overall	69.5 \pm 1.7 c	64.2 \pm 2.6 b	61.6 \pm 3.2 ab	59.2 \pm 3.7 a

a,b,c different: $P < 0.05$.

^a Modified extender Long-Term.

^b Modified extender Short-Term.

^c Medinova-Reggio nell'Emilia.

^d IMV-France.

extended semen at 36, 72 and 166 h of storage in all the trials. An AI trial was then performed in two different breeding farms practicing integral pig production with similar management conditions (feeding levels, breeding, health status and environmental conditions) using their own boars, on a total of 1011 sows: 506 using the short term modified extender (ME-S) and 505 with the commercial extender (CRONOSTM). Both extenders were tested in each farms. After oestrus check, the sows were inseminated two times in 24 h and in case a 3rd insemination at 36–72 h, if immobility reflex was still present. The insemination dose was 3×10^9 sperms in 100 ml volume with 80% of sperm motility in fresh diluted semen and 75% when doses were stored. The motility was checked by visual scoring with a light microscope for both extenders. The first AI was performed with fresh diluted semen, while the second and the third with the semen stored at 16 °C. Hormonal therapy was not used to induce ovulation in the sows. Diagnosis of pregnancy was performed by ultrasound techniques at 25–26 days after AI. Pregnancy rate and piglets born alive were considered and recorded. The data relevant to motility were statistically analyzed by ANOVA (SAS, 2008) using a linear model including the fixed effects of the type of extender (4 levels), time (7 levels), trial (12 levels), boar (15 levels) and interaction between extender and time. Data are reported as least squares means (\pm SE) of the interaction between extender and time. Data from the AI trial were analyzed by χ^2 test (Kaps and Lamberson, 2009). Piglet data were analyzed by ANOVA using a linear model with type of extender (2 levels) and parity order of sows (3 levels: 1st, 2nd and > 2nd parity) as fixed effects.

3. Results

The main peculiarity regarding the composition of the two modified extenders is the use of amikacin sulphate and fructose (Table 1), instead of gentamicin and glucose, that are present in the commercial extender (CRONOSTM). Table 2 shows that motility was not different ($P > 0.05$) among extenders until 120 h, as differences among extenders became significant ($P < 0.05$) at 144 and 166 h.

Table 3

Comparison between the reproductive efficiency of the new short-term modified extender (ME-S) and the commercial extender (CRONOSTM).

Extender	No. of sows	Pregnancy rate no. (%)	No. of piglets born alive/sow (mean)
ME-S ^b	506	474 (93.68%)	11.78
CRONOS ^a	505	452 (89.50%)	12.24
χ^2 (P)		5.71 (0.0169)	(n.s.)

n.s.: not significant ($P > 0.1$).

^a CRONOS (MedinovaTM, Reggio nell'Emilia, Italy).

^b Modified extender Short-Term (ME-S).

In particular, ME-L and ME-S showed higher motility than TRIXcellTM at 144 h and than CRONOSTM at 166 h. At 24 and 48 h of storage CRONOSTM seems to show higher motility if compared to ME-S, but the differences are not significant ($P > 0.05$). Motility data across time were better in ME-L and ME-S than in TRIX-cellTM ($P < 0.05$). No differences were observed about the morphology and membrane integrity (ORT.) between the new extenders and commercial ones. Microbiological investigation showed the presence of *Serratia marcescens*, *E. coli*, *Proteus spp.*, *Pseudomonas aeruginosa*, *Staphylococcus spp.* and *Streptococcus spp.*, according to data reported in literature Maroto Martín et al., (2010). The new modified extenders, as a powder, did not show any physical (flow and packing) and organoleptic (colour) variation compared to the commercial one (CRONOSTM), which tended to become brown and clotted with time. The results regarding the biological trial are reported in Table 3. The pregnancy rate was higher for ME-S extender (93.68%) compared to a commercial short-term extender (89.50%) ($P < 0.05$). The mean number of piglets born alive was not significant ($P > 0.05$). Nevertheless the total number of piglets born alive was 0.9% higher in ME-S than in CRONOSTM (5584 piglets vs. 5532), due to the higher number of pregnant sows.

4. Discussion

In swine industry great emphasis is put on improving fertility results (Broekhuijse et al., 2012). The most important result obtained with the new modified extender (ME-S) was the increase of pregnancy rate, with a total increase of 52 more piglets in ME-S group than in CRONOSTM group. According to result it can be affirmed that in breeding farms with a large number of sows, this increase could improve the owner income. Moreover, in our study, differences in fertility data between ME-S and CRONOSTM can be referred only to extender characteristics, because the other factors that normally affect fertility (Broekhuijse et al., 2012) were homogeneous in the farms selected for the field trial. The effect of the farm was not included in the statistical model because farms were similar for management system, feeding levels, geographic location and also for the breed of sows. Since adding an antibiotic at the appropriate concentration improves sperm survival and, in turn, fertility (Colenbrander et al., 1993), the main reason of these results is the presence of a high dosage of amikacin sulphate compared with the amount of gentamicin

in the commercial extender (four time less). In our opinion the presence of amikacin sulphate improves semen reproductive efficacy because of better control of bacterial growth in the extender. In fact boar ejaculates tend to be unavoidably contaminated with bacteria during semen collection process. Metabolic by-products of uncontrolled bacterial growth leads to pH fluctuations or other bacterial products are responsible for the reduction in sperm longevity and herd fertility or they have directly spermicidal effects (Althouse, 1997; Althouse and Lu, 2005). Bacterial contamination of porcine semen has been associated with deleterious effects of semen quality (Althouse et al., 2008) affecting motility and structure of the sperm, causing sperm agglutination (Monga and Roberts, 1994), leading to lower fertility, conception rates, litter size at birth (Maroto Martín et al., 2010). Gentamicin has been added to several boar semen extenders since 90s, but yet in 1997 Kuster and Althouse reported the presence of gentamicin-resistant bacteria in extended boar semen, and following studies found the presence of contaminant bacteria resistant to gentamicin (Althouse and Lu, 2005; Althouse et al., 2000). The ME-S amikacin concentration did not show any deleterious effect on sperm viability. Amikacin, which showed a good activity against isolated bacteria, in our opinion improved semen quality reducing bacterial contamination, in particular during field conditions. It should be kept in mind that extenders are very reach media that contain enough nutrients to support bacterial growth (Maroto Martín et al., 2010). We could also postulate that amikacin could have positive effects on genital tract of the inseminated sows; further studies on this topic are needed. In the new modified extenders the energy source has been substituted by fructose instead of glucose, since it is naturally present in boar seminal plasma. In contrast to results reported in literature (Gadea, 2003), the presence of fructose led to appreciable sperm motility for short term purpose storage. In fact results of percentage sperm motility at 96 and 120 h of storage for ME-L showed are not acceptable to be used for AI. The composition of the formulation was selected after a deep investigation of the compatibility of the components in order to assure high physical and chemical stability. Thereafter the powders were evaluated for physical (flow and packing) and organoleptic (colour) properties, in order to guarantee uniform composition. Actually, we could suggest that fructose at the dosage used for our study is too quickly metabolized by spermatozoa. Further investigations are needed to improve ME-L motility results during storage. For the AI trial only the ME-S has been used. Nowadays, in Italy a concern is that AI doses older than 48 h may lead to fertility losses, particularly in terms of litter size, as discussed in literature (Christensen et al., 2004). When the storage time is lower than 72 h, the use of short-term extenders is preferred because they are cheaper and the reproductive results are similar compared to long-term extenders. Therefore, it seems that even using a short-term extender, good preservation of certain semen characteristics, such as sperm membrane integrity and chromatin stability, is provided (De Ambrogi et al., 2006). When the storing time is over 4 days (long distances, diseases control, etc.), a long-term extender and a higher sperm concentration must be used to compensate the loss of spermatozoa viability by ageing. So the selection of

the semen extender must be done to optimize the reproductive results (fertility and litter size) according to the conditions of each porcine farm (Gadea, 2003).

5. Conclusions

From the results obtained in the present study, we can conclude that: the new modified extender ME-S provided a good semen motility for short-time storage; the increase of pregnancy rate is higher compared with the commercial one, since the pregnancy rate increases.

Conflict of interest

The author's disclose any actual or potential conflict of interest including any financial personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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