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# Effect of Different Extenders and Washing of Seminal Plasma on Buck Semen Storage at 5°C

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**Abstract:** In this research, we compared the effect of three extenders for buck semen conservation; skimmed Milk (M), sodium Citrate (C) and a Tris-based diluent (T) and the washing of semen (removal of seminal plasma) on the *in vitro* viability of Murciano-Granadina goat spermatozoa stored at 5°C for 72 h. Motility, acrosome integrity and HOS test were evaluated to assess *in vitro* sperm viability. Milk diluent provided higher *in vitro* viability of spermatozoa than semen diluted in T during storage at 5 °C. Motility in semen diluted in citrate and milk extenders was improved when semen was washed previously. In milk extender, membrane integrity (HOST) was also improved with the washing of semen. In conclusion, removal of seminal plasma could be necessary for successful chilled conservation of buck semen at 5°C when M or C based diluents is used. Milk media and washing of seminal plasma appears to be a successful method to prolong the viability of Murciano-Granadina goat semen stored at 5 °C. The latest results must be confirmed in field assays.

**Key words:** Goat, chilled semen, extender, seminal plasma, milk media, memberanwe integrity

## INTRODUCTION

Regardless frozen semen in cervical artificial insemination could reach acceptable fertility results in goat (55-65%, Leboeuf *et al.*, 1998; Salvador *et al.*, 2005), the utilization of cooled semen is an important alternative to be considered in a breeding programme, especially when the most of the animals live in a small geographical area. A correct progeny test requires a great number of inseminations, although this number is reduced when fertility increases. Fertility using semen stored at room temperature or cooled at 5°C is higher than when frozen semen is used (Ritar and Salamon, 1983; Roca *et al.*, 1997; Leboeuf *et al.*, 1998; Paulenz *et al.*, 2005), so by using cooled semen the number of inseminations required to test the future males could be reduced.

Nowadays, the generally used media for buck semen liquid storage (4-5°C) are skimmed milk, sodium citrate-egg yolk and Tris-egg yolk diluents (Leboeuf *et al.*, 2000). Removal of seminal plasma by washing buck semen has been reported to increase the percentage of live spermatozoa and their motility during storage in egg yolk or milk diluents (Corteel, 1974; Ritar and Salamon, 1982; Leboeuf *et al.*, 2000). However, in Murciano-Granadina goat, Roca *et al.* (1997) did not obtain any improved effect of washing seminal plasma on *in vitro* viability or *in vivo* fertility of chilled semen diluted in Tris extender. Differences in the effect of washing seminal plasma in semen conservation may rely on factors such as breed, season, buck, diluent, breed of fowl providing the egg yolk and intensity of the washing procedure (Corteel, 1973; Ritar and Salamon, 1982).

To our knowledge, there is no work in the literature comparing the effect of different extenders and its interaction effect with the process of washing of seminal plasma in Murciano-Granadina goat semen preserved at 5°C.

The aim of the present research was to study the effect of three different extenders: milk, sodium citrate-egg yolk and Tris-egg yolk and the effect of washing of seminal plasma on the *in vitro* survival of Murciano-Granadina goat spermatozoa stored at 5°C.

## MATERIALS AND METHODS

**Animals and semen collection:** Semen collection was performed following Silvestre *et al.* (2004). Briefly, semen was collected by artificial vagina from seven 12 to 24 month-old Murciano-Granadina bucks, under uniform nutritional conditions. Semen collection was always performed by the same technician. Immediately following collection, the ejaculates were immersed in a warm water bath at 30°C until their assessment in the laboratory. Semen assessment was performed in approximately 20 min.

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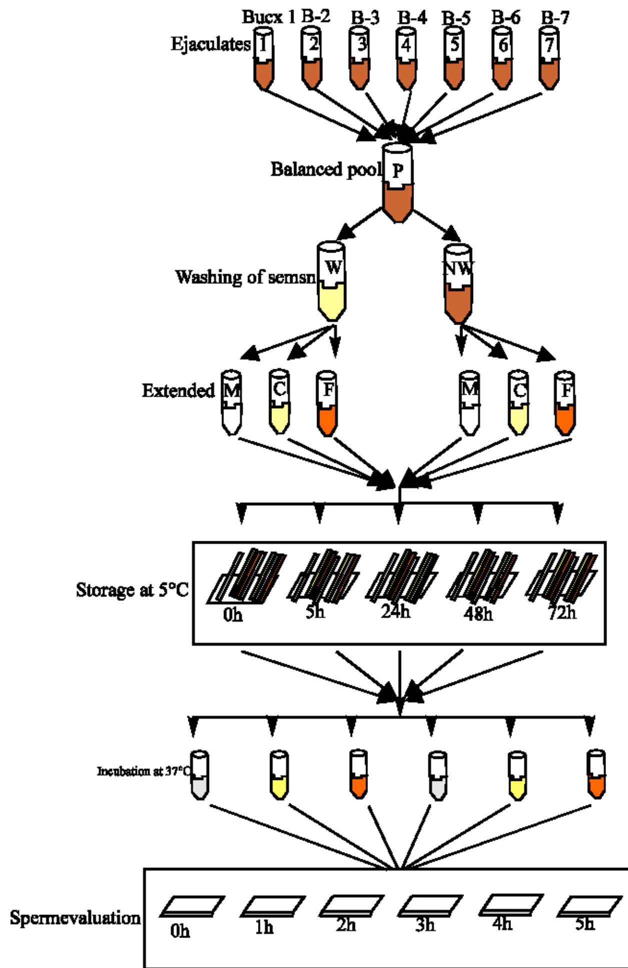


Fig. 1: Pool was split in two equal volumes, Washed (W) and No Washed (NW). After washing, both pools were split and diluted in Milk (M), Tris-based (T) and Citrate-based (C) based extender, respectively. Semen was stored at 5°C for 0, 6, 24, 48, 72 h. One straw from each treatment was evaluated for sperm quality (motility; membrane integrity: HOST and acrosome: NAR) and motility was further evaluated every hour of incubation (0, 1, 2, 3, 4, 5 h) at 37 °C

**Extenders**

**Three extenders were tested:** *Milk (M)*: Milk-based extender was composed of long-life Ultra-Heat-Treated (UHT) skimmed-milk and prepared following Corteel (1974) protocol. Briefly, 10 g of skimmed-milk powder was diluted in 100 mL water containing 0.2 g D-glucose, heated in a water bath at 92° C for 10 min and cooled at room temperature.

***Tris-Citric-Glucose-Yolk (T)*:** Tris-based extender was prepared following E vans and Maxwell (1987), so that concentrations in diluted semen were: 250 mM of Tris, 82.7 mM of Citric Acid and 27.7 mM of glucose. Final egg yolk concentration in diluted semen was 12 and 2% (v v $^{-1}$) in washed and non washed semen, respectively.

***Citrate-Glucose-Yolk (C)*:** Citrate-based extender was also prepared following Evans and Maxwell (1987), so that concentrations in diluted semen were: 60.4 mM of Sodium Citrate and 33.3 mM of glucose. Final egg yolk concentration in diluted semen was 12 and 2% (v v $^{-1}$) in washed and non washed semen, respectively.

T and C extenders were stored frozen and thawed each day of session to be used. M extender was prepared the day before each session.

**Semen preparation:** Volume of ejaculates was measured in a conical tube graduated at 0.1 mL intervals and concentration was determined by a spectrophotometer (Acucell, IMV, France) calibrated for goat species (1:400 dilution rate). Only those ejaculates surpassing motility values of 75% (evaluation described below) and sperm

concentration of $3\times10^9$ spermatozoa $mL^{-1}$, were mixed in a pool balancing the sperm contribution of each male (Paulenz *et al*., 2002).

The pool of semen was split in two equal volumes, one of them to be removed from its seminal plasma by washing and the other one maintaining its seminal plasma. Washing of seminal plasma was performed following Salvador *et al.* (2006). Briefly, semen was washed by centrifugation during 10 min at 900 g, diluted in Tris based media without egg yolk (1/10 vol. semen/vol. Tris).

After washing procedure, both pools (washed and no washed) were diluted with the above described extenders to $600\times10^6$ spermatozoa $mL^{-1}$, packaged in 0.25 mL straws and stored at 5° C.

**Evaluation of sperm samples**

**Sperm motility:** Subjective sperm motility was assessed by diluting an aliquot of semen in sodium Citrate solution (C) without egg yolk and under a phase-contrast microscopy system (100X) with a warmed plate at 37° C.

**Thermal resistance test:** Thermal Resistance Test (TRT) was performed by incubation of spermatozoa in Tris-based media (1/4 dilution) at 37° C (in a water bath) after a previous light washing of the extender by centrifugation (5 min at 600 g diluted 1/4 in Tris-based media). One sample from each treatment was evaluated after 0, 1, 2, 3, 4, 5 h of incubation to determine sperm motility.

**Acrosome integrity (NAR):** Sperm acrosome integrity was assessed only at 0 h incubation time, by measuring percentage of spermatozoa population with Normal Acrosome (NAR). We diluted (1/100) 10 µL of semen sample in a NaCl (0.9%) solution with 2% of glutaraldehyde and, under phase-contrast microscopy (1000X), percentage of spermatozoa population with non reacted and normal acrosome was measured.

**Osmotic resistance test (HOST):** HOST was also performed only at 0 h incubation time, by adding an aliquot of semen (10 µL) in 100 µL of hypo-osmotic solution (100 mOsmol $kg^{-1}$: 1 g sodium citrate in 100 mL water). Assessment was performed after 1 h of incubation at 37°C under phase-contrast microscopy (400X) and percentage of spermatozoa population with swollen tail was scored.

**Experimental design:** A complete 3×2×5 factorial design was used to compare three commonly used extenders for fresh buck semen conservation (M, T, C) in washed or non washed semen (W, NW) up to 72 h of storage (0, 12, 24, 48, 72 h). Five replications were performed. The whole semen processing procedure is illustrated in Fig. 1.

**Statistical analysis:** Analysis of Variance (ANOVA) of the data was performed using the General Linear Model (GLM) in the Statgraphics Plus 4.1 Software (Manugistics Inc., Rockville, MD). We included the effect of extender, washing, storage time, incubation and all single interactions in the model.

## RESULTS

Significance for the effects of extender, washing of semen, storage time, incubation and all single interactions on motility, acrosome integrity and hyposmotic test are shown in Table 1. Extender and storage time affected all sperm parameters evaluated ($p<0.001$) and washing of semen affected only motility ($p<0.001$) and HOST ($p<0.01$). Motility was affected ($p<0.001$) by incubation during TRT.

Differences within the evaluated sperm parameters (motility, NAR and HOST) between extenders and washing of semen are indicated in Table 2. All the sperm parameters in semen diluted in Tris extender were significantly lower than in semen extended with milk and citrate ($p<0.05$). Semen motility in milk extender was significantly higher than in citrate and Tris ($p<0.05$). NAR and HOST of semen diluted in citrate or milk extender were no different. Washing of semen improved motility and HOST ($p<0.05$) whereas it did not significantly improve NAR. Semen diluted in Tris extender showed a steeper

Table 1: Significance based on GLM for the effects of extender, washing, storage time, incubation and all the interactions on subjective Motility (MOT), Acrosome integrity (NAR) and Hyposmotic Test (HOST)

| | Sperm viability parameters[a] | | |
|---|---|---|---|
| Source of variation | MOT | NAR | HOST |
| Washing of semen | *** | Ns | ** |
| Extender | *** | *** | *** |
| Storage time | *** | *** | *** |
| Washing×extender | *** | Ns | * |
| Extender×Storage | *** | ** | *** |
| Incubation | *** | | |
| Washing×incubation | Ns | | |
| Extender×incubation | Ns | | |
| Storage×incubation | Ns | | |

[a] NAR and HOST assessments were only performed at 0 h, only motility was assessed in TRT (incubation). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; Ns: not significant

Table 2: Least square means (%) for subjective Motility (MOT), Acrosome integrity (NAR) and Hyposmotic Test (HOST) for the different factors studied[a]

| | Sperm viability parameters | | |
|---|---|---|---|
| Source of variation | MOT | NAR | HOST |
| Extender | | | |
| -Milk 62 a | 65 a | 39 a | |
| -Citrate 55 b | 66 a | 37 a | |
| -Tris 45 c | 60 b | 25 b | |
| Washing of semen | | | |
| -Washed 58 a | 65 a | 35 a | |
| -Non washed | 50 b | 63 a | 33 b |

[a]Least square means in columns and within each source of variation, without letters in common, are significantly different ($p<0.05$)

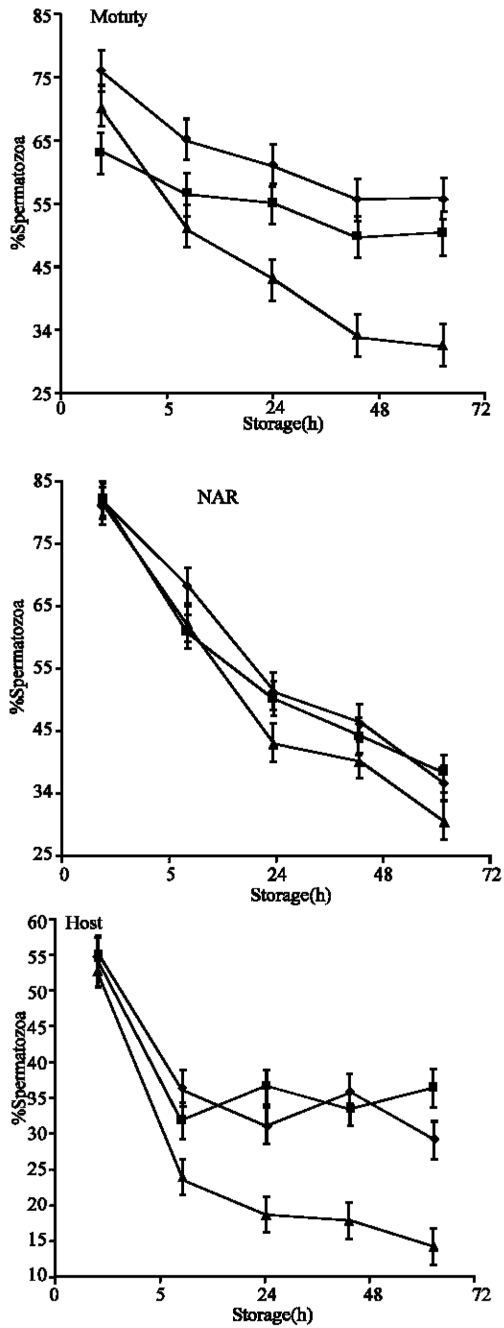


Fig. 2: Least square mean interval of percentage of subjective motile (motility), normal acrosoma (NAR) and osmotic resistant (HOST) spermatozoa of fresh pooled semen from seven bucks, diluted in three different extenders ((?) M; (|) C; (?) T) and evaluated after 0, 5 , 24, 48 and 72 h of storage at 5°C

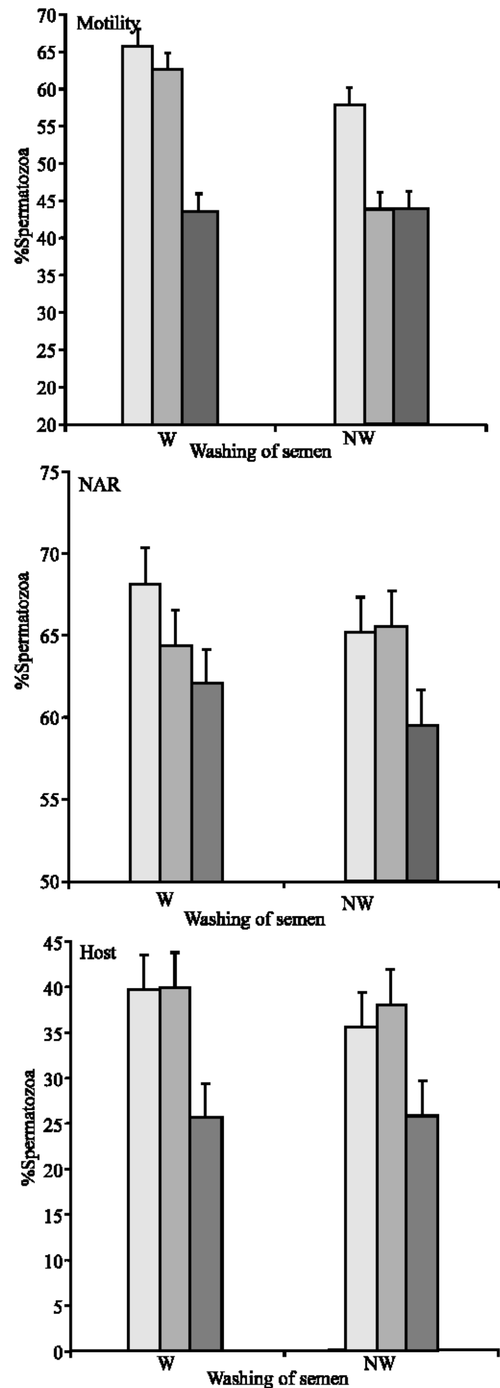


Fig. 3 Least square mean interval of percentage of subjective motile (motility), normal acrosoma (NAR) and osmotic resistant (HOST) spermatozoa of fresh pooled semen from seven bucks, washed (W) or not washed (NW) to eliminate seminal plasma and diluted in three different extenders ((|) M; (\\\\\) C; (||||||) T). Data pooled from evaluations after 0, 5, 24, 48 and 72 h of storage at 5°C (Exp.1).

decrease with storage time in sperm motility and HOST, whereas in NAR, it only showed a significant decrease at 24 storage time, in comparison with citrate and milk extenders (Fig. 2). Semen diluted in Tris extender was not affected by washing in any of the sperm parameters evaluated. However, sperm motility was higher when semen was washed before dilution in citrate and milk extender and HOST was also improved by washing, but only in milk extender (Fig. 3).

## DISCUSSION

In the present research we studied the effect of three extenders for liquid semen storage (Milk, Citrate and Tris based extenders) on *in vitro* survival of Murciano-Granadina goat spermatozoa stored at 5°C. Under our conditions, milk extender appeared to provide higher *in vitro* spermatozoa viability, whereas semen diluted in Tris based extender had the lowest *in vitro* survival during storage at 5°C. In contrast with our results, Shamsudddin *et al.* (2000) observed that Black Bengal buck semen motility remained at 50 % or more after chilling for 4 days in Tris-fructose-egg yolk (2.5%, v $v^{-1}$) diluent, 3 days in citrate-glucose-egg yolk (2.5%, v $v^{-1}$) diluent and only 2 days in skimmed milk diluent. Palomino *et al.* (2001) reported that, under refrigeration at 4°C, semen diluted in citrate-egg yolk (20%, v $v^{-1}$) maintained good motility for 96 hours, while that prepared with skimmed milk-egg yolk (20%, v $v^{-1}$) ceased to function after 48 h. It must be emphasised that in these latter works semen was not washed. In our case, up to 72 h, milk extender preserved motility better than Citrate and Tris extenders in non washed semen (2%, v $v^{-1}$ egg yolk) and also better than Tris-glucose-egg in washed semen (12%, v $v^{-1}$ egg yolk). The variability in the performance of the diluting media to preserve goat semen is not surprising in the light of the interactions of seminal plasma with egg yolk and constituents of milk media. The toxicity of Egg Yolk Coagulating Enzyme (EYCE) differs with the quantity of hydrolysates, which varies with pH, temperature, seminal plasma concentration, season of semen production, breed, buck and breed of fowl providing the egg yolk (reviewed by Leboeuf *et al.,* 2000).

In buck semen, removal of seminal plasma by washing the spermatozoa in a physiological solution has been proved to increase the percentage of live cells and their motility during storage in egg yolk (Ritar and Salamon, 1982) or milk diluents (Corteel, 1974). Certain enzymes in the seminal plasma, originating from the bulbourethral gland secretion, catalyse the hydrolysation of egg yolk lecithin and milk triglycerides of the extender, releasing sperm-toxic products (lysolecithin and fatty acids) that lead to subsequent sperm deterioration (Roy, 1957; Iritiani *et al.*, 1961; Nunes *et al.,* 1982; Pellicer-Rubio and Combarnous, 1998). Ritar and Salamon (1982) recommended, however, the use of = 1.5% egg yolk in Tris-based diluent without removal of seminal plasma as a practical alternative in frozen semen, estimating 1.5% egg yolk concentration as the safe margin, at least in the Tris based diluent, to avoid any depressing effect on viability of spermatozoa even of those bucks ‘sensitive’ to higher egg yolk levels. In accordance with the latter authors, in our study when Tris extender was used, unwashed semen (2% egg yolk) maintained its viability as well as performed by washed semen (12% egg yolk). However, motility of sperm diluted in Milk and Citrate extenders were improved when semen was washed previously. There are discrepancies about the beneficial effect of the removal of seminal plasma on sperm survival during storage in the literature. Roca *et al.* (1997) did not obtain any improved effect of washing seminal plasma on *in vitro* viability of chilled semen diluted in Tris extender with 2 and 12% egg yolk concentration for unwashed and washed semen, respectively, whereas Islam *et al.* (2006) obtained beneficial effect of removal of seminal plasma on the quality of goat semen (crossbred Beetal×Assam Local) during preservation at 5°C up to 72 h in Tris extender (20% egg-yolk).

Membrane integrity (HOST) in semen diluted in Tris showed a deeper decrease during storage time than in semen extended in Milk or Citrate. HOS test is an indication of sperm membrane resistance and is positively correlated with results of *in vitro* oocytes fertilization with fresh semen (Hauster *et al.,* 1992) and Hamster test (Fuse *et al.,* 1991).

Differences in the response of the sperm membrane to hypotonic stress in each treatment (extender×storage time and extender×washing) could be related to modifications of the membrane composition and/or structure during survival (Leboeuf *et al*., 2006). HOST parameter showed partially the same pattern as those observed in motility as reported by Leboeuf *et al.* (2006).

## CONCLUSION

Removal of seminal plasma could be necessary for successful long-term chilled conservation of buck semen at 5°C when Milk or Citrate based diluents are used. Milk media and washing of seminal plasma appears to be a successful method to prolong viability of Murciano-Granadina goat semen stored at 5 °C. Further field assays are necessary.

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