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ORIGINAL ARTICLE

# The effect of citric acid and citrate on protoplasmic droplet of bovine epididymal sperms

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## Abstract

For evaluation of citric acid and citrate effects on bovine epididymal protoplasmic droplets, fifty bovine testes were collected in the October 2007 till June 2008 from Urmia slaughterhouse and transported to the laboratory in a cool container filled with 5 °C ice pack. Caudal epididymis was incised and sperm cells were put into Petri dishes containing hams f10 media with 10% fetal calf serum (FCS), which were kept in 37 °C, $CO_2$ incubator. Then sperm cells were counted and 50-milion per mL concentration was prepared. After this stage, three dilutions of citric acid (0.1, 0.2, 0.3 N) and one dilution of citrate (1N), based on normal osmolarity and normal pH were added to a micro tube containing 25 milion per mL sperm. Then one-step eosin-nigrosin staining in 30-60-120-240-360 minutes was performed and slides were evaluated with 1000-x phase contrast microscope and 200 sperm cells per slide were counted. The results revealed significant difference between blank and citric acid 0.3 N. The proportion of protoplasmic droplet in group consisting of 0.3 N acid citric in 120-240-360 minutes, was significantly lower than that of blank ($P < 0.05$). There was no significant difference between citrate – blank and citric acid 0.1N-blank groups, but after 240 minutes significant difference was observed between blank & citric acid 0.2 N ($P < 0.05$). In conclusions citric acid based on dilution and time duration can reduce the proportion of bovine epididymal sperm cytoplasmic droplets.

## Introduction

Following spermatogenesis and maturation during epididymal transit, spermatozoa are stored in the caudal epididymis and remain until the time of ejaculation.[1]

In particular cases, epididymal sperm are the only available source of male gametes for use in assisted reproduction programs. For example, this can be the case when spermatozoa have to be urgently retrieved from a severely injured or suddenly deceased donor, and can be vitally significant with a donor belonging to an endangered species. Cattle breeding and selection programs have illustrated that the introduction of beef and dairy breeds in certain parts of the world has led to the near extinction of native breeds; for instancein West Africa, Madagascar and Brazil. This results in dire consequences, because the available native gene pool is valuable and irreplaceable to generate crossbred cattle adapted to local climate conditions, poor quality feed and endemic diseases.[1]

In some situations, superior males have to be handled or captured under anesthesia, and this might impair normal ejaculation.

Moreover, theoretically, an ejaculate can be devoid of spermatozoa, which makes aspiration of epididymal sperm,or a testicular biopsy to recover sperm, necessary.[1]

However, the unexpected death of animals of high genetic value or zoological interest, as well as the difficulty in collecting semen from wild species, is a handicap to the application of assisted reproduction techniques for preservation of biodiversity. The recovery and freezing of viable sperm from the epididymis of dead animals (post-mortem recovery) is an interesting option for sperm recovery is a useful strategy for germplasm banking .This technique allows to use the epididymal sperm reserves of deceased or hunted males, especially when semen collection would be difficult or impossible by other ways.[3]

Sperm stored in the caudal epididymis have usually good quality and a high level of maturation, being able to fertilize oocytes.[4] To date, many studies have demonstrated that it is possible to obtain viable gametes in postmortem. Furthermore, successful pregnancies have been achieved in many species using epididymal sperm for artificial insemination.[5-8] Epididymal sperms have protoplasmic droplet.[9] Thus, it has been reported that more than 60% of spermatozoa from the caudal epididymidis have a droplet, each in bulls, goats and boars.[9] It is suggested, however, that the droplets are shed from goat spermatozoa during their transit through the urethra and/or immediately after ejaculation, and the results obtained in bulls have revealed that the percentage of spermatozoa with the droplet noticeably was decreased in the ampulla of the ductus deferens[9] and it is reported that in ampulla citric acid is secreted into the seminal plasma.[10]There are no enough available data on the effects of different levels of citric acid or citrate doses, *in vitro* on bull's epididymal spermatozoa. Citric acid, in bull's vas deferens ampulla, is secreted into the seminal plasma naturally.[10]

In this study, three levels of citric acid ( 0.1,0.2,0.3 N) and one level of citrate(1N) based on normal seminal plasma pH and normal osmolarity were used (1N=720 mg 100 $mL^{-1}$ of seminal plasma).[8,11,12]

## Materials and Methods

**Sample collection.** For this experiment testes of 25 mature bulls between the ages of 18 and 24 months were collected in the October 2007 till June 2008 in Urmia slaughterhouse and 50 gonads were transported to the laboratory in a cool container filled with 5 °C ice pack, one hour after males death. Spermatozoa were collected from the distal portion of the epididymis according to the method of Soler *et al.*[13] In brief, spermatozoa were recovered from the distal portion of the epididymis by cutting the caudal epididymis with a scalpel, collecting the oozing sperm mass and placing it in 1 mL of Ham's F10 media (between 285-295 miliosmol) containing 10% FCS (Fetal Calf Serum).[13]

The sperm mass was then diluted again at room temperature to a final sperm concentration of ≃50 milion sperm/mL which were kept in 37 °C, $CO_2$ incubator (sperm cells counted with Direct Cell Count [Hemocytometer]. No. sperm/mL = no. sperm in 0.1 $mm^3$ × l0 × dilution rate × 1,000 ).[12]

Three dilutions of citric acid (0.1, 0.2, 0.3 N or, 0.07, 0.14, 0.21 mg $mL^{-1}$) and one dilution of citrate (1N,7 mg $mL^{-1}$) were added to micro tube which in final was consisting of 25 million sperm cells per mL.

### Sperm staining.

**The one step eosin-nigrosin staining technique.** The staining solution for the one-step technique was contained 0.67% eosin Y (Certistain CI 45380 K476951 887 VWR No.1.15935 Merck) and 10% nigrosin (CI 50420, preparation, 0.67 g of eosin Y and 0.9 g sodium chloride were dissolved in 100 mL distilled water under gentle heating. Then 10 gr of nigrosin was added, after cooling in room's temperature, the solution was filtered and kept in 37 °C $CO_2$ incubator till staining (Fig 1-8).

The one-step eosin-nigrosin staining technique was performedin 30-60-120-240-360 minutes after incubation and slides were evaluated with 1000× phase contrast

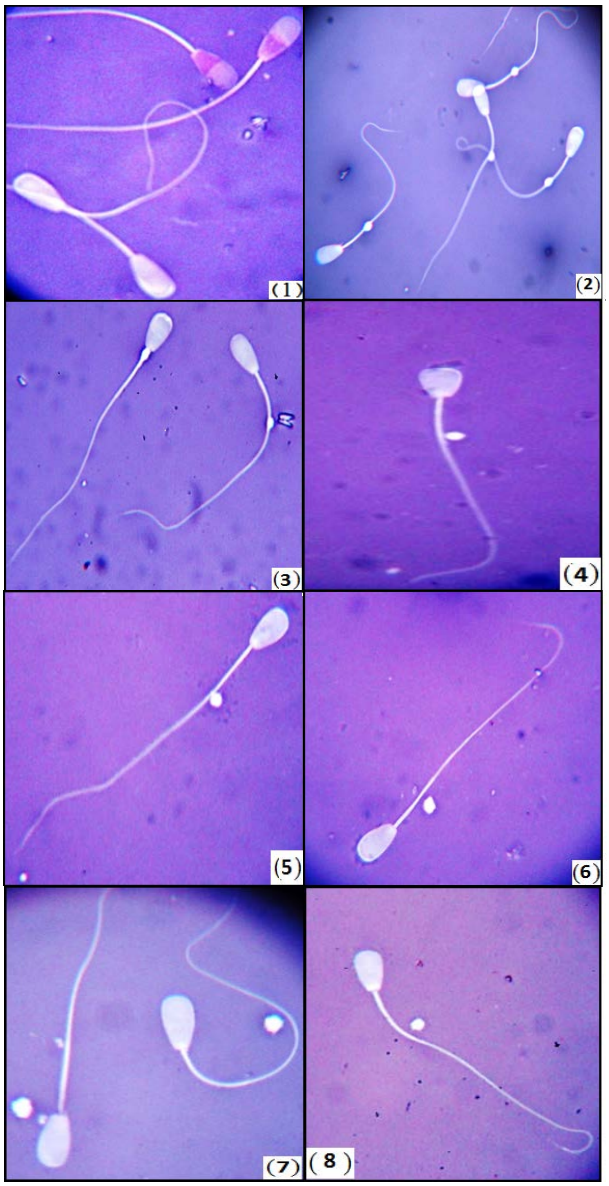


**Fig 1**. Live and dead sperm cells (eosine-nigrosine. 1000×)
**Fig 2.** Live epididymal sperms with distal droplets (eosine-nigrosine. 1000×)
**Fig 3.** Live epididymal sperms with distal and proximal droplets (eosine-nigrosine. 1000×)
**Figs 4 & 5.** Live epididymal sperm with loosed droplet (eosine-nigrosine. 1000×)
**Figs 6, 7 & 8.** Live epididymal sperm with separated droplet (eosine-nigrosine. 1000×)

microscope and 200 sperms per slide were counted.[14] Then percentages of live/dead and protoplasmic droplets evaluations were carried out.

**Statistical analysis.** The obtained data were analyzed using one-way analysis of variance of SPSS ver15 and significant values were compared using Tukey's multiple range test.

## Results

No significant differences were observed between values until 60 minutes, but there were significant differences between blank and 0.3N citric acid in 120, 180, 240 and 360 minutes, i.e. proportion of protoplasmic droplets in the group consisting of 0.3 N citric acid was lower than that of the blank ($P < 0.05$). There was no significant difference between citrate–blank and 0.1N citric acid–blank, but in 180, 240, 360 minutes, significant differences were observed between 0.3N citric acid and 0.1N citric acid, i.e. proportion of protoplasmic droplets in the group consisting of 0.3N citric acid was lower than that of the 0.1N ($P < 0.05$) (Table 1).

Table 2 shows the result of live-ratio percentage of epididymal sperm cells and no differences were observed between values among different groups and different minutes ($P > 0.05$).

## Discussion

Sertoli cells absorb the cytoplasm of a spermatid during spermiation. The remnant of the cytoplasm, known as the "cytoplasmic droplet", is located on the cytoplasm, known as the "cytoplasmic droplet", is located on the neck of the spermatozoon.[9,15] This remnant structure has been variously known as a kinoplasmic droplet,as it was considered to have something to do with motility, a cytoplasmicbead, a protoplasmic droplet, a cytoplasm droplet and a plasma droplet, but is generally known today as a cytoplasmic droplet.[15,16] Cytoplasmic droplets (proximal droplets: surrounding the neck and proximal midpiece of sperm; distal droplets: surrounding the midpiece just proximal to the annulus) are spherical masses of cytoplasm 2–3 μm in diameter found on the tails of sperms.[16,17] However, it has been clearly established that mammalian spermatozoa leaving the testis contain a cytoplasmic droplet. On leaving the testis, the cytoplamic droplet is located in the region rear to the head of the spermatozoon.[16] All spermatozoa possess a proximal droplet as they pass into the caput epididymis. It has been reported that spermatozoa undergo final maturational changes, including acquiring the ability to fertilize and translocation of proximal droplets to the annular region (distal droplet), during transit through the epididymis. It has been suggested that regional changes in the ionic composition of epididymal fluids may be responsible for the distal movement and ultimate loss of droplet. It has also been shown that a hemolytic factor, phospholipids binding protein (PBP) in seminal vesicular fluid causes the release of the distal droplet from bull sperm. It has been suggested that fructose originating from seminal vesicle stimulates shedding of distal cytoplasmic droplets in boar sperm.[16]

Subsequently, the proximal droplet moves posteriorly along the midpiece until it reaches the point of annulus, [17] during proximal epididymal transport and remains on the

**Table 1.** The percentage of protoplasmic droplets (Mean ± SEM).

| | Times (minutes) | | | | | |
|---|---|---|---|---|---|---|
| Groups | 30 | 60 | 120 | 180 | 240 | 360 |
| Blank | 64.67 ± 5.04 | 64 ± 4.73 | 63.34 ± 4.81 $^{a}$ | 63 ± 5.13 $^{a}$ | 62.32 ± 5.24 $^{a}$ | 61.33 ± 5.70 $^{a}$ |
| Citric Acid (0.1N) | 63.33 ± 4.81 | 62 ± 5.13 | 60 ± 4.58 $^{ab}$ | 58.33 ± 4.18 $^{ac}$ | 57 ± 4.04 $^{ac}$ | 55.33 ± 3.67 $^{ac}$ |
| Citric Acid (0.2N) | 62 ± 4.58 | 60 ± 4.73 | 58.33 ± 4.70 $^{ab}$ | 55 ± 4.16 $^{ab}$ | 51.67 ± 4.67 $^{ab}$ | 48.33 ± 4.63 $^{ab}$ |
| Citric Acid (0.3N) | 60.33 ± 4.41 | 52.33 ± 2.91 | 41.33 ± 2.73 b | 39.33 ± 3.28 $^{b}$ | 37 ± 3.06 $^{b}$ | 33.16 ± 2.3 $^{b}$ |
| Citrate (1N) | 61.33 ± 4.70 | 58.67 ± 4.48 | 56 ± 5.13 $^{ab}$ | 51.67 ± 3.18 $^{ab}$ | 49.67 ± 3.76 $^{ab}$ | 48.67 ± 3.76 $^{ab}$ |

Values with dissimilar superscripts in each column are significantly different ($P < 0.05$).
As indicated in table1, the results show significant differences in 120,180,240 and 360 minutes between blank and 0.3 N citric acid The percentage of protoplasmic droplets of epidimyal sperm cells in 0.3 N citric acid are significantly less than that of the blank group ($P < 0.05$).There are also significant differences in,180,240 and 360 minutes, between 0.3 N and 0.1 N citric acid and the percentage of protoplasmic droplets of epidimyal sperm cells ,in 0.3 N are significantly less than that of the 0.1N citric acid ($P < 0.05$). In other groups, there were no significant differences ($P > 0.05$).

**Table 2**. The live sperm ratio (Mean ± SEM).

| | Times (minutes) | | | | | |
|---|---|---|---|---|---|---|
| Groups | 30 | 60 | 120 | 180 | 240 | 360 |
| Blank | 77.39 ± 3.76 | 83.93 ± 3.26 | 76.48 ± 4.24 | 71.21 ± 2.63 | 71.48 ± 1.78 | 69.14 ± 0.99 |
| Citric Acid (0.1N) | 75.98 ± 3.53 | 81.31 ± 4.22 | 77.48 ± 4.34 | 72.64 ± 4.17 | 71.14 ± 1.62 | 70.75 ± 1.46 |
| Citric Acid (0.2N) | 77.72 ± 3.62 | 80.45 ± 3.70 | 77.08 ± 3.20 | 76.73 ± 4.75 | 77.37 ± 1.81 | 75.51 ± 1.80 |
| Citric Acid (0.3N) | 79.3 ± 4.81 | 81.61 ± 5.35 | 82.11 ± 3.85 | 83.07 ± 5.23 | 84.37 ± 1.94 | 78.95 ± 2.41 |
| Citrate (1N) | 85.32 ± 1.31 | 77.55 ± 3.15 | 79.01 ± 1.74 | 74.25 ± 5.05 | 71.97 ± 1.01 | 74.62 ± 2.93 |

majority of cells in the epididymis.[9]

The mechanism of transport has not been elucidated but migration of droplets from the neck of caprine and porcine testicular spermatozoa can be achieved by the application of repeated or sustained centrifugation.

Peristaltic motions of the epididymal tubule acting on high concentrations of spermatozoa within the lumen could thus contribute to the migration of the droplet along the tail.[9]

In bulls producing normal semen, distal droplets are shed when sperm are exposed to seminal plasma during ejaculation.[16] It has been reported that the droplet can be artificially shed from an epididymal spermatozoon or disintegrated by rough procedures, smearing of sperm samples before the fixation and changes in the pH of the sperm suspension.[15]In our experiments, the sperm samples were collected gently from the organs immediately after removal from the animals without contamination of blood and tissue debris, and were fixed immediately. The ampulla of the ductus deferens is well developed in bulls. The results obtained in bulls have revealed that the percentage of spermatozoa with the droplet strikingly were decreased in the ampulla of the ductus deferens and, the concentrations of cations ($Na^+$, $Ca^{2+}$ and $Mg^{2+}$) and citric acid were much higher in fluid from the ampulla than from the epididymis.[15] Shedding of droplets from boar spermatozoa occurs within 1 min after ejaculation. It is suggested, however, that the droplets are shed from goat spermatozoa during their transit through the urethra and/or immediately after ejaculation. Phospholipid-binding protein (hemolytic factor), which appears to be associated with the shedding of droplets from spermatozoa, is secreted from the ampulla and the seminal vesicle in bulls.[15]

The lysosomal enzymes of the droplets perhaps prepare the spermatozoon for the final stage of its maturation. It is assumed that the event of gradual reduction in the shape and size of the cytoplasmic droplets plausibly were accompanied by dehydration of the vesicle which is surrounded by plasmalemma that resulted in ultra-structural changes of the droplets,[18] on the other hand regional alterations in the ionic composition of the epididymal fluids are believed to be responsible for distal movement and ultimate loss of the droplet.[18].The results of the present study confirmed significant differences in 120,180,240,360 minutes between blank and 0.3N citric acid groups. The percentage of protoplasmic droplets of epidimyal sperm cells in 0.3N citric acid was less than that of blank group ($P < 0.05$).

In conclusion, citric acid could reduce the percentage of protoplasmic droplets of bovine epididymal sperms. By now the real mechanism of separation of distal protoplasmic droplet has been unclear and few mechanisms including the present study have been proposed, however main reason or causes remain to be unclear and more researches should be done. Acrosomal integrity, sperm motility parameters and fertility rates of the sperms must be discussed after separation of the droplets has been performed

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