

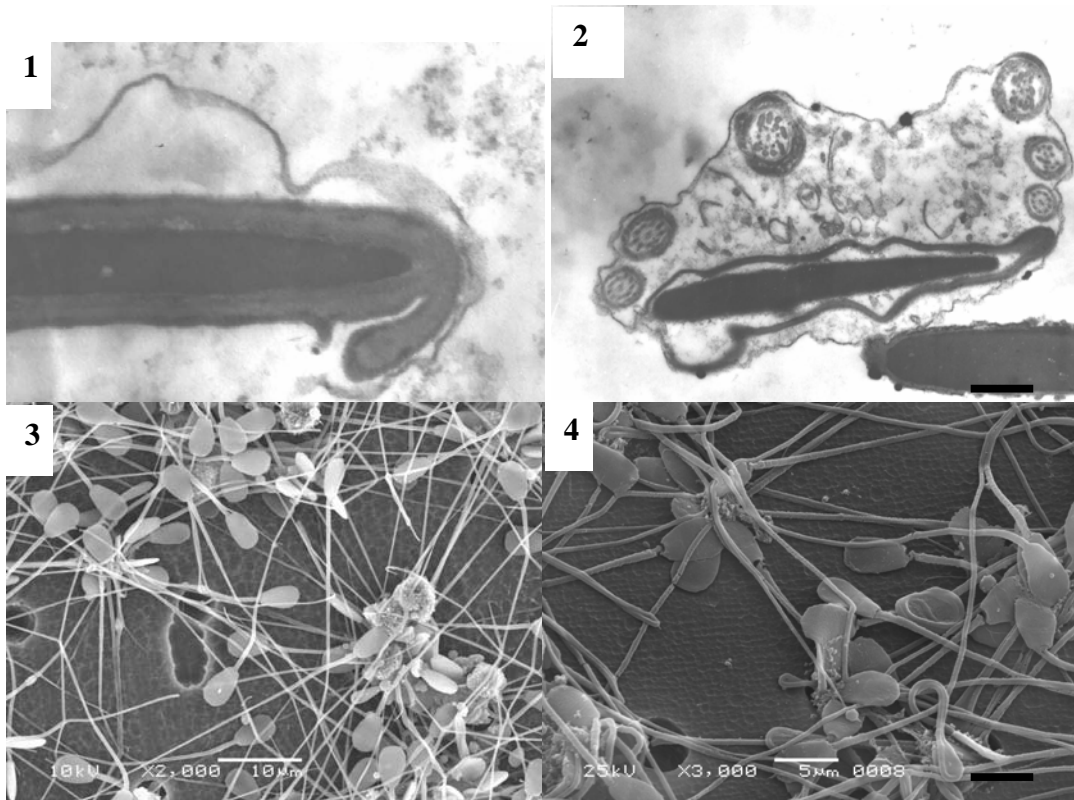
## ULTRASTRUCTURAL ANALYSIS OF FRESH AND FROZEN-THAWED DOG SPERMATOZOA.

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Many extenders have been studied for canine semen cryopreservation. Tris-based extenders are commonly used in both practical and experimental situation and are known to give good pregnancy rates (1). Compounds containing sodium dodecyl sulphate (SDS) alone or as one component in Equex STM paste have been included in extenders used for canine semen cryopreservation (1,2). Likewise, different kind of sugar had been included in extenders used for canine semen cryopreservation (1, 2, 4, 11). Cellular membranes, including those of sperm, must withstand a variety of stresses during freezing and thawing. According this sperm membranes are affect by cryopreservation (10) and the plasmatic membrane is considered to be the site at which freezing induced injury is initiated (6). Acrosome, cytoskeleton, motile apparatus and nucleus are to involved in frozen-thawed damage (10). These deleterious effects were demonstrable using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The aims of this study was to characterize the type of ultrastructural changes present in canine sperm after freezing-thawing using trehalose and Equex STM paste in a Tris extender or Tris extender. Ultrastructural changes were study in frozen-thawed dog spermatozoa diluted in Tris egg yolk extender with 5% glycerol (extender A), extender A containing 1.5% of Equex STM, extender A containing 5% and 7% of trehalose. Semen was collected by digital manipulation from three (n=3) fertile German Shepard dogs. The sperm rich fraction was divided into four parts. Each part was diluted in each of the different extenders in a two step dilution before equilibration (2) to a final sperm concentration of  $100 \times 10^6$ /ml and was packed in 0.5 ml straws. The straws were frozen in a styrofoam box 4 cm above liquid nitrogen (5). For TEM one sample was prepared immediately after collection. The other samples were taken after freezing-thawing for Tris and Equex extender. All samples were centrifuged and fixed in 3% glutaraldehyde in PBS buffer and postfixed with 1% osmium tetroxide in PBS buffer. The sections were stained with uranyl acetate and lead citrate and examined in a JEM 1200 EX II. For SEM one sample was prepared immediately after collection. The other samples were taken after freezing-thawing for Tris, Equex and trehalose extenders. All samples were fixed in 1% glutaraldehyde and filtered in a 5  $\mu$ m diameter Millipore membrane. Then, specimens were dehydrated, critical-point dried and coated with gold palladium. Samples were observed using a JSM 6360 LV. Ultrastructural study whit TEM showed in fresh samples, most spermatozoa had a normal appearance, although some abnormalities were observed: acrosome lipping, macrocephalic, nuclear vacuoles, multiple tails surrounded by a common membrane, "Dag" defect. After freezing-thawing spermatozoa showed several ultrastructural changes: plasmatic membrane was disruption, swollen and presented plica formations. Different kind of damage acrosomal such as swelling of the acrosome and unevenly distributed acrosomal content, vesiculation of the outer acrosomal membrane and the plasmatic membrane and detachment of the acrosome were observed (Figs. 1, 2). Ultrastructural study whit SEM showed in fresh samples, most spermatozoa had a normal appearance (Fig. 3), although some abnormalities were observed: distal protoplasmic droplet, coiled tail. After freezing-thawing in samples frozen whit extender A many spermatozoa with acrosome damage, broken neck and detached tail was observed. Semen frozen whit extender A including trehalose showed many spermatozoa with intact acrosome but with broken tails. When semen was frozen with extender A including Equex STM paste we observed many intact cells but some spermatozoa with acrosome damage and intact tail was detected (Fig. 4). Freezing of dog spermatozoa may have both immediate and delayed effects on the ultrastructure of spermatozoa. Our TEM finding corresponds with those previously obtained from others researches (7, 8, 9). The cryoprotective action of trehalose has been explained by its dehydrating activity and interaction with cell membranes. Likewise, STM paste improves the cold shock protection by modifying the structure of egg yolk lipoprotein. The damage observed in frozen-thawed spermatozoa was in relationship with the type or locality of protective impact of compounds included in the extender. This fact can be observed in the sample analyzed by SEM. Further studies are warranted to test the fertilizing ability and pregnancy rates following artificial insemination using canine frozen-thawed semen diluted with these extenders.

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**Fig. 1-** TRIS extender. Acrosome lipping. Bar= 200 nm.

**Fig. 2-** DAG defect. Bar= 500 nm.

**Fig. 3-** Fresh semen. Bar= 10  $\mu$ m.

**Fig. 4-** Equex 1,5% extender. Bar= 5  $\mu$ m.