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# Ultrastructural alterations of canine spermatozoa treated with styrene maleic anhydride co-polymer *in-vitro*

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**Abstract:**
*A morphological change in sperm cell is considered to be a potential target in contraceptive development. In the present study, the physical and ultrastructural characteristics of canine spermatozoa treated with Styrene Maleic Anhydride (SMA) co-polymer in vitro were evaluated using Scanning Electron Microscopy (SEM). Semen collected from dogs (n=6) of different breeds by digital manipulation were diluted with Tris buffer for microscopical examination. Semen from each sample was divided into two aliquots viz. group A, treated with co-polymer SMA [(2mg of SMA dissolved in 100 µl of Dimethylsulfoxide (DMSO)] and group B, served as sham control sample which received DMSO only. After 60 min of incubation, the mean (± SE) motility percentage of SMA co-polymer treated group and DMSO control group of canine spermatozoa was 4.83 ± 1.01 and 53.33 ± 0.95, respectively. Similarly, the mean (± SE) acrosomal damage percentage of SMA co-polymer treated and DMSO control groups were 13.16 ± 1.71 and 49.96 ± 1.02, respectively. SEM examination showed ultrastructural alteration in acrosomal, mid piece and tail region in SMA co-polymer treated sperm cells, while no such changes were detected in DMSO control group as well as in pretreated canine spermatozoa. The results of the present study clearly indicate the loss of functional competence of canine spermatozoa on in vitro treatment with SMA co-polymer.*

**Key words:** *Canine, spermatozoa, in vitro, styrene maleic anhydride, scanning electron microscopy*

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## I. Introduction

Sperm morphology is regarded as a significant prognostic factor for fertilization and pregnancy. Since, abnormal sperm morphology is the most common factor causing male infertility, inducing morphological changes in sperm is considered to be a potential target in contraceptive development. Styrene Maleic Anhydride (SMA) has been demonstrated to be one such co-polymer that provides pH lowering effect and charge disturbances to the sperm membrane, resulting in acrosomal damage, which thus brings about deleterious effect to spermatozoa (Guha, 1999). As these specific sperm alterations are not identifiable by conventional methods of semen analysis, it is important to explore the damage caused by SMA using scanning electron microscope (SEM).

The SEM is a valuable and useful technique for ultrastructural investigation of sperms of infertile animals. It remains distinct in its ability to examine dimensional topography and distribution of exposed features

of sperms. Moreover, optical morphological investigation is not an adequate technique, because expected changes might not be revealed with it. In addition, evaluation of the morphology is subjective, leading to considerable error in the estimation (Grab *et al.*, 1993). As expected changes may be of the order of submicron or at the nanometer scale and, therefore, it demands ultrastructural investigation by using SEM. Hence, the present study was carried out to evaluate changes in the physical and ultrastructural characteristics of canine spermatozoa treated with co-polymer Styrene Maleic Anhydride (SMA) *in vitro* using Scanning Electron Microscopy.

## II. Materials and Methods

***Chemicals:*** DMSO and Tris were obtained from Sigma-Aldrich (USA), while Styrene Maleic Anhydride co-polymer was prepared by Radical addition-fragmentation chain transfer (RAFT) polymerization technique at Translational Research Platform for Veterinary Biologicals,TANUVAS, TamilNadu (India).

***Semen collection and evaluation***

Semen was collected from six healthy and sexually matured dogs of different breed, aged between 2-6yrs by digital manipulation. Immediately after collection the semen sample was transferred to the laboratory and recorded for its volume, colour and consistency. Further, the sperm rich second fraction was subjected to microscopical examination for motility, concentration, viability and acrosomal integrity by diluting with Tris [tris(hydroxymethyl)aminomethane] buffer solution. Semen from each sample was divided into two aliquots viz. group A considered as experimental fraction, where in sperm cells were treated with co-polymer SMA [(2mg of SMA dissolved in 100 µl of Dimethylsulfoxide (DMSO)] and group B, served as sham control sample which received DMSO only. Both the groups were incubated *in vitro* for 60 min. based on the standardization as the optimum time for significant damage of membrane and analysed for its physical and ultrastructural changes by using SEM.

***Scanning Electron Microscopic (SEM) analysis of spermatozoa***

Semen samples were centrifuged for 5 min. at 500 r.p.m. and washed twice with phosphate buffer (0.1mol/L ; ph 7.2) for 5 min. each. After washing, the samples were centrifuged at 500 r.p.m. for 5 min. After removing the supernatent fluid, the recovered pellet was immediately fixed in 2.5% glutaraldehyde in phosphate buffer for 30 min. and washed again in phosphate buffer solution three times for about 5 min. each.The samples were dehydrated in 70% ethanol for 30 min. and thin film of spermatozoa was smeared on a SEM stub with silver paint, air dried and mounted on sputter coated with gold palladium and observed under Scanning Electron Microscope (TESCAN VEGA 3 SBU,CIECH Republic country).Statistical analysis was done as per standard technique described by Snedecor and Cochran (1994).

### III. Results

***Semen analysis by light microscope***

The Physical, morphological and functional characteristics of fresh dog semen are presented in table 1. In the present study, sperm motility of freshly collected semen was more than 60 per cent in all the donor dogs. Light microscopic study revealed that all semen parameters viz. sperm motility, sperm concentration, live and abnormal percentage were within normal range.

***Ultrastructural examination of spermatozoa by SEM***

Scanning electron microscopic evaluation of morphological and ultrastructural characteristics of sperm cell from pre-treated semen sample showed oval head with distinct acrosomal and post acrosomal region. The acrosomal region was smooth and intact. Neck of the sperm cell appeared as a slight constriction and connecting the head and midpiece. The midpiece and tail showed intact mitochondrial and fibrous sheaths (Fig. 1).

***In vitro characterization studies of canine spermatozoa***

The spermicidal action of the SMA co-polymer was tested *in-vitro*, using canine ejaculated semen. After 60 min of incubation, the spermatozoa from group A and B were subjected to microscopical examination for motility and acrosomal integrity assessment as well as ultrastructural analysis by SEM (Fig. 2).

In the present study, the mean (± SE) motility percentage of SMA co-polymer treated group and DMSO control group of canine spermatozoa was 4.83 ± 1.01 and 53.33 ± 0.95, respectively. Similarly, the mean (± SE) acrosomal damage percentage of SMA co-polymer treated and DMSO control groups were 13.16 ± 1.71 and 49.96 ± 1.02, respectively. The morphological and ultrastructural changes in canine spermatozoa on treatment with SMA co-polymer and DMSO were examined using Scanning Electron Microscopy (SEM). Sperm cells from SMA treated groups showed rough and damaged acrosomal region (Fig. 3A). Mid piece with loss of sperm membrane and disintegration of mitochondrial sheath (Fig. 3B), while coiled tail with damaged

sperm membrane were visualized (Fig. 3C and 3D). In DMSO control group, sperm cells with smooth surfaced acrosomal region, intact mid piece and tail membranes were noticed. In the present study, ultrastructural alteration in acrosomal, mid piece and tail region was observed in SMA co-polymer treated sperm cells, while no such changes were detected in DMSO control group as well as in pretreated canine spermatozoa.

## IV. Discussion

In mammalian sperm cells, it is well known fact that the destabilization of the plasma membrane along with the underlying outer acrosomal membrane system leads to vesiculation which subsequently causes leakage of molecules necessary for fertilization (Bhattacharyya and Zaneveld, 1982; Chaudhury *et al.,* 2004; Sunil kumar *et al*., 2006). The acrosomal membrane needs to be intact until the spermatozoa attaches to the oocyte and any disturbance in the membrane releases enzyme required for degradation of the cumulus matrix (George Anifandis *et al*., 2014).The present study is an attempt to understand the action of SMA co-polymer on *in vitro* canine spermatozoa using SEM. Significant changes were observed in the acrosomal and midpiece region of spermatozoa treated with SMA in vitro. These results were in accordance with earlier studies on membrane integrity of human sperms in presence of RISUG (Chaudhury *et al.,* 2004). While, Lohiy *et* al., (1998a and1998b) reported a significant damage to the acrosome and midpiece of langur monkeys ejaculates when injected with RISUG.

In the present study, loss of sperm motility by the addition of SMA co-polymer was probably related to the presence of carboxylic group. Polymer containing carboxylic groups was found to be strong inhibitor of sperm motility. Singh *et al*. (1984) in their studies on effects of synthetic polymers on the motility of human spermatozoa found that 100 per cent immotile spermatozoa were observed after 120 min of treatment with poly (SMA). They stated that the poly (SMA) as such did not affect the motility of the spermatozoa but with time, the maleic anhydride group hydrolysed to maleic acid which inhibited the motility of spermatozoa.

Any alteration in the cytoskeletal structure of the sperm flagellum as evident from the SEM studies could be a contributing factor for the severely reduced or completely absent sperm motility. In the present study, damage to plasma-membrane of canine spermatozoa leading to tail coiling was noticed when treated with SMA co-polymer *in vitro*. Our findings concurred with Chemes (1991 and 1993) that SMA caused significant structural changes in the morphology of the sperm tail leading to its coiling and thus rendering it infertile. Similarly, complete disintegration of the plasma membrane with subsequent rupture and dispersion of acrosomal content were observed in the human sperms treated with RISUG *in vitro* (Sunil kumar *et al*., 2006). Such damage to the acrosomal and midpiece membrane was due to polyelectrolytic nature of SMA co-polymer in inducing surface charge imbalance on sperm membrane system which leads to rupture of sperm heads and results in leakage of enzymes necessary for fertilization (Guha, 1999 and Chaudhury *et al*., 2004). One of the techniques that is capable of detecting even discrete structural changes of sperm cell membranes is Scanning Electron Microscopy. Much work has been done in recent years on the evaluation of fine structure of human, monkey, rabbit, sambar deer spermatozoa (Colone *et al.,* 2017; Wilborn *et al.,* 1983; Mahre *et al.,* 2014). However, no such work on evaluating infertility has been reported in canine spermatozoa. Hence, in the present study damage to the sperm cell membrane caused by treating dog spermatozoa with SMA *in vitro* had been discussed and also recorded that SMA could able to induce spermicidal effect on canine spermatozoa *in vitro.*

## V. Conclusion

In conclusion, the morphological and ultrastructural alterations occur dramatically in head and midpiece region of canine spermatozoa treated with SMA co-polymer. Future, it will pave the way for development of non hormonal male contraceptive agent in canine.

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**Table:** Semen characteristics of donor dogs

| Semen parameters | Mean ± SE |
|---|---|
| Volume of second fraction (ml) | 1.62 ± 0.17 |
| Colour | White to milky white |
| Consistency | Thin to medium |
| pH | 6.20 ± 2.40 |
| Motility (%) | 75.50 ± 1.10 |
| Sperm concentration ($x10^6$ spermatozoa/ml) | 412.47 ± 20.24 |
| Live spermatozoa (%) | 83.66 ± 1.28 |
| Abnormal spermatozoa (%) | 13.84 ±0.68 |
| Acrosomal integrity (%) | 74.16 ±2.44 |

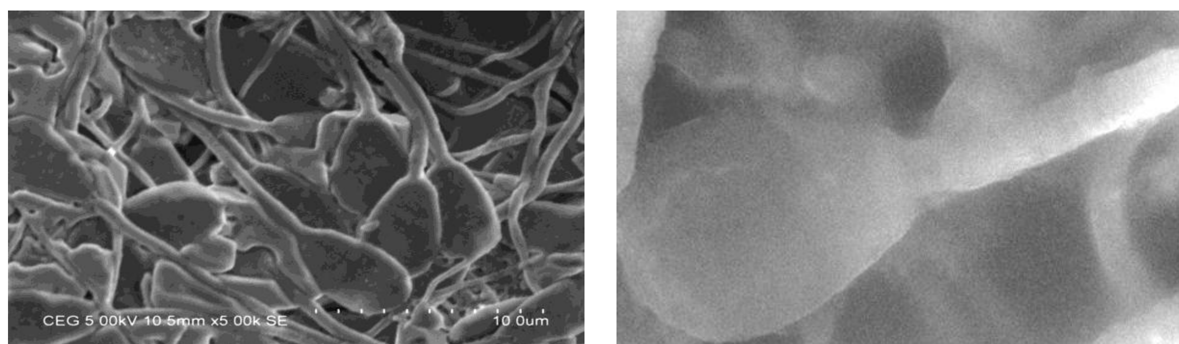



**Figure1**. Scanning electron micrographs of pretreated canine spermatozoa in clusters (10 µm) and normal sperm head(3µm)

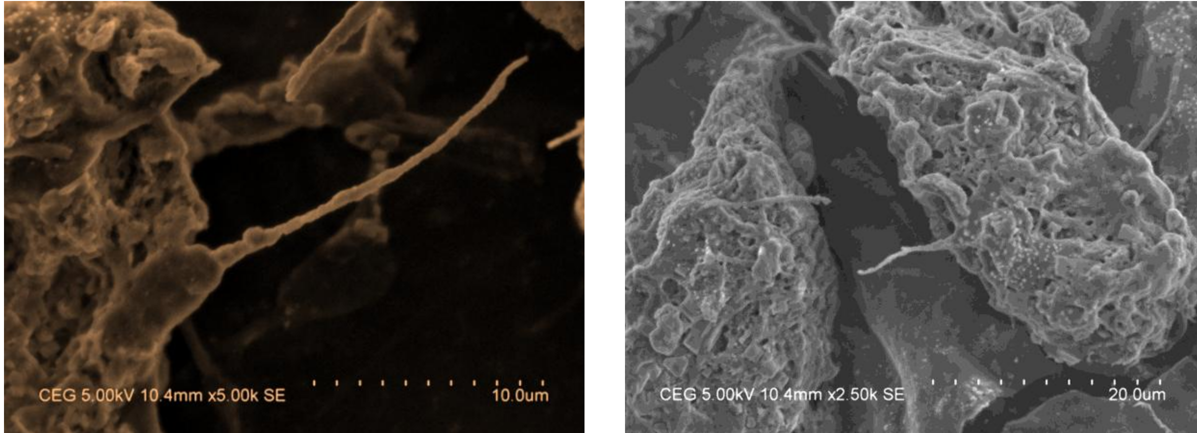



**Figure 2**. Scanning electron micrographs of canine spermatozoa trapped in SMA polymer in 10µm and 20µm magnification

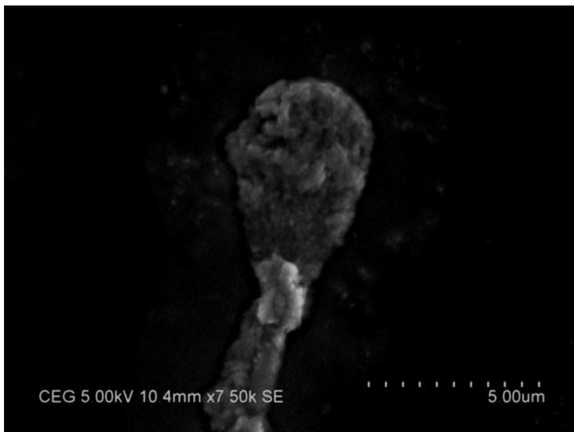



**Figure 3A**. Scanning electron micrographs of canine spermatozoa showing deformed, ruptured heads with thickened and damaged mid piece (5µm)

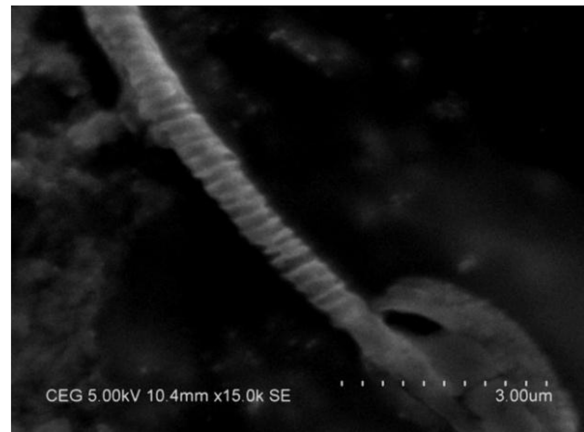
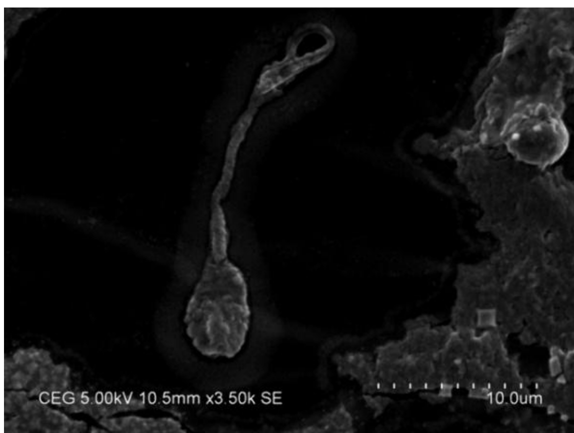



**Figure 3B**. Scanning electron micrographs of canine spermatozoa showing damaged mitochondrial sheath (3µm)



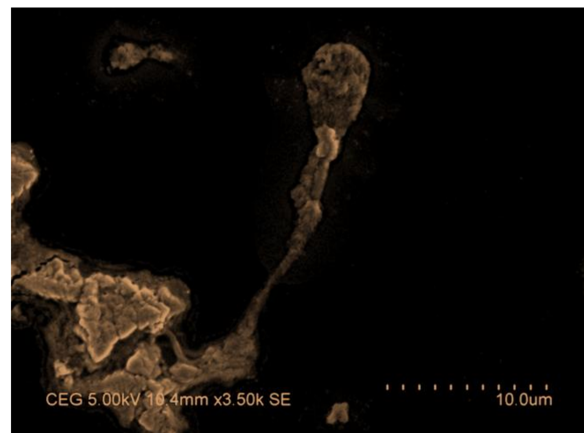




**Figure 3C and 3D**. Scanning electron micrographs of canine spermatozoa showing damaged head, mid piece and tail(10µm)

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