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Scanning Electron Microscopic Studies of Frozen Fowl Semen

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(Figs 1–20; Table 1)

Recently the scanning electron microscope (SEM) is currently being used to study the morphology of normal and abnormal spermatozoa. In general, it is known that the acrosome of mammalian spermatozoa is affected by deep freezing preservation.[1)–3)] On the other hand, HARRIS *et al.*[4)] also reported that the acrosome of fowl spermatozoa is influenced by deep freezing as well as in that of mammalian spermatozoa. MARQUEZ *et al.*[5)] reported that some swelling or distortion of the mitochondria were caused when the turkey semen has been exposed to a glycerol containing medium. But there are few reports pertaining to the series of the morphological changes in fowl spermatozoa after freezing and thawing.

In the present study, the scanning electron microscope was used to observe the morphological changes observed in fowl spermatozoa before, during and after freezing and thawing of sperm cell.

MATERIALS AND METHODS

The semen used for the present experiment was collected from 3 White Leghorn cockerels of 10 months old and mixed. The mixed semen was diluted to four fold with a 5.7% glucose solution, and a 5.7% glucose solution 85 plus fresh egg yolk 15 which contained 7% glycerol in final concentration. After dilution, the semen samples were frozen for one hour by two freezing methods; one the pelleted freezing method[6)] and the other the straw freezing method[7)] described in the previous papers. The pre-freezing time of semen samples in straw freezing was 3 minutes. Afterwards, those samples were thawed in 5°C and 37°C respectively. The smear preparation of fresh undiluted semen for studies of spermatozoa by the light microscope was fixed in formalin vapour and stained with carbol-fuchsin-eosin by the routine staining procedure of our laboratory. The fixation of fresh and thawed semen samples for studies of spermatozoa by SEM were carried out for an hour at 4°C with glutaraldehyde solution (2–4%). After the samples were washed two times with a phosphate buffer, the samples were smeared on aluminum-foil and then air-dried. Afterwards, the smear samples were dehydrated in an ethanol for 30 minutes and were coated with gold palladium. Then the morphological changes of specimens were observed. Motility of samples was scored by a scale of five point (+++, ++, +, ±, –). For estimating the percentage of abnormal spermatozoa, approximately 500 spermatozoa were examined.

RESULTS AND DISCUSSION

MOTILITY AND ABNORMALITY OF FOWL SPERM CELL

The motility and abnormality of undiluted semen and that of the semen samples treated by two freezing methods (pelleted freezing method and straw freezing method) with two diluents (5.7% glucose solution and 5.7% glucose solution 85 plus fresh egg yolk 15) are shown in Table 1. The percentages of motility and abnormal spermatozoa of undiluted semen calculated as the control were 95% (over ++) and 2.9% respectively.

Table 1. Motility and abnormality of fowl spermatozoa after thawing.

Freezing method	Pelleted freezing method		Straw freezing method	
Diluent	5.7% glucose solution	5.7% glucose solution plus fresh egg yolk	5.7% glucose solution	5.7% glucose solution plus fresh egg yolk
Motility after dilution	95	95	95	95
Motility after thawing	70	80	80	80
Abnormality after thawing	7.8	8.4	10.3	11.1

Notes: Motility of fresh undiluted semen 95% (++ over)
Abnormality of fresh undiluted semen 2.9%

As shown in Table 1, the motility of semen samples after thawing by two freezing methods (pelleted freezing method and straw freezing method) and two diluents (5.7% glucose solution and 5.7% glucose solution 85 plus fresh egg yolk 15) are pretty active ranging from 70 to 80% (over ++) in each division. The abnormalities of the thawed semen which were treated by the above two freezing methods and two diluents were 8.1% on the average ranging from 7.8 to 8.4% in the former and 10.8% on the average ranging from 10.3 to 11.1% in the latter respectively. These results are higher than 5% in the former and 7% in the latter compared to those of the undiluted semen (2.9%). But these results are not always as high as in the result of freezing semen.

MORPHOLOGY OF FOWL SPERM CELL

1. Observation by the light microscope

The morphology of fresh semen by the light microscope is shown in Figs. 1 and 2. The distortion of the midpiece could rarely be seen in Fig. 1 but it was seen in the central field of Fig. 2.

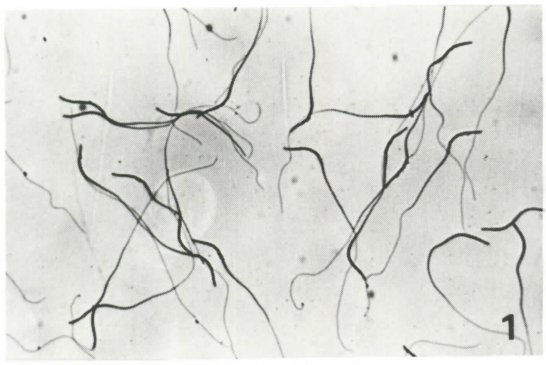

Fig. 1. Micrograph of fresh semen immediately after collection by the light microscope (LM). Abnormal spermatozoa are rarely seen in this micrograph. LM X400.

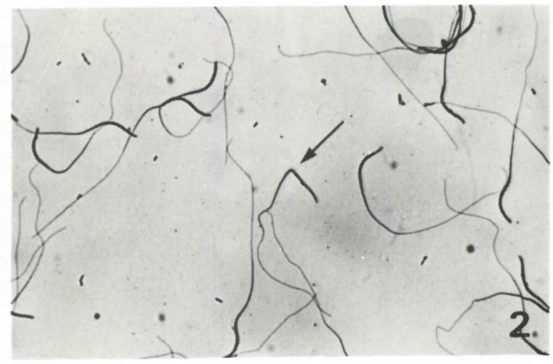

Fig. 2. Micrograph of fresh semen immediately after collection by LM. Neck bending of sperm cell is seen in the central field of this figure. LM X400.

This distortion of the midpiece is called "neck-bending of sperm"[8], "bent spermatozoa"[9] or "crooked-necked spermatizoa".[10] This phenomenon in sperm cells of fresh semen was observed in present fresh semen. (Fig. 2) According to TSUKUNAGA *et al.*[8], neck-bending of sperm is a response of the live sperm cell to its hypotonic medium; any hypotonic solution whose freezing-point depression is lower than $-1.03°C$, owns this particularity to its osmotic pressures. Since the $\triangle$ of the cock's seminal plasma consisting of transparent accessory sex secretions is much lower than this, showing only $-0.58°C$, it is no wonder that even fresh undiluted semen exhibits neck-bent sperms gradually increasing in number with the elapse of time.

The micrographs of the spermatozoa after the thawing by means of pelleted freezing and the two diluents (5.7% glucose solution and 5.7% glucose solution 85 plus fresh egg yolk 15), are shown in Figs. 3 and 4. The micrographs of those thawed by straw freezing and two diluents (5.7% glucose solution and 5.7% glucose solution 85 plus fresh egg yolk 15) are shown in Figs. 5 and 6.

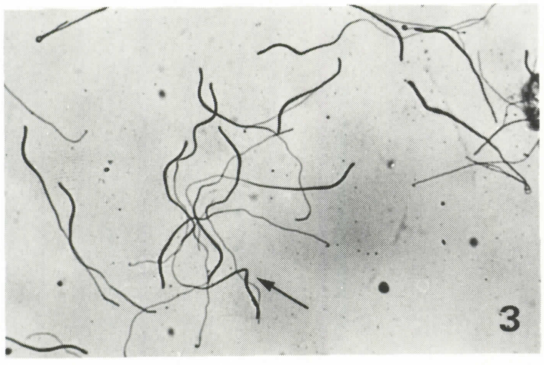

Fig. 3. Micrograph of the spermatozoa diluted with 5.7% glucose solution by pelleted freezing method. In this micrograph, neck-bending spermatozoon is seen. LM X400.

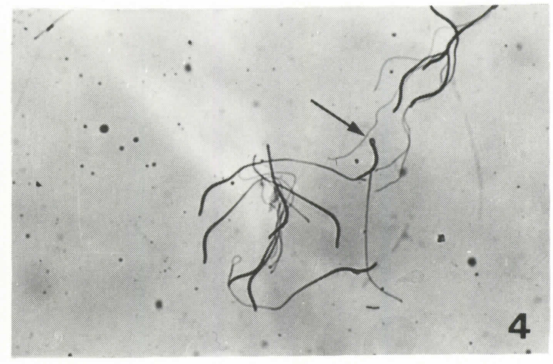

Fig. 4. Micrograph of the spermatozoa diluted with 5.7% glucose solution plus fresh egg yolk after thawing by pelleted freezing mthod. In this micrograph, neck-bending spermatozoon is seen. LM X400.

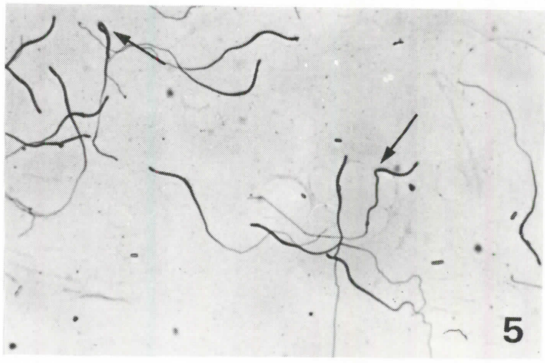

Fig. 5. Micrograph of the spermatozoa diluted with 5.7% glucose solution after thawing by straw freezing method. Neck-bending spermatozoa are seen in this figure. LM X400.

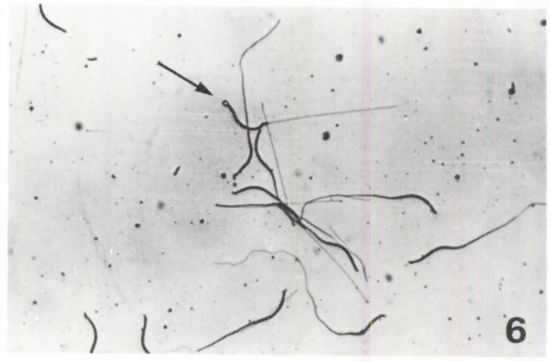

Fig. 6. Micrograph of the spermatozoa diluted with 5.7% glucose solution plus fresh egg yolk after thawing by straw freezing method. Neck-bending spermatozoon is seen. LM X400.

In each micrograph, the neck-bending of the sperm can be seen notwithstanding the freezing method or the diluent but the differences of the occurrence of the neck-bending of the sperm could not be seen among them. Moreover, as there is a credit limit in the light microscope, the detailed observation of sperm cells, especially that in the sperm head could not be obtained. Therefore, we have tried out further observations on sperm cells using SEM.

2. Observations under the scanning electron microscope

The ultrastructures of the undiluted (non-frozen) fowl spermatozoa are shown in Figs. 7 (X 1,000), 8 (X 2,000), 9 (X 5,000) and 10 (X 10,000).

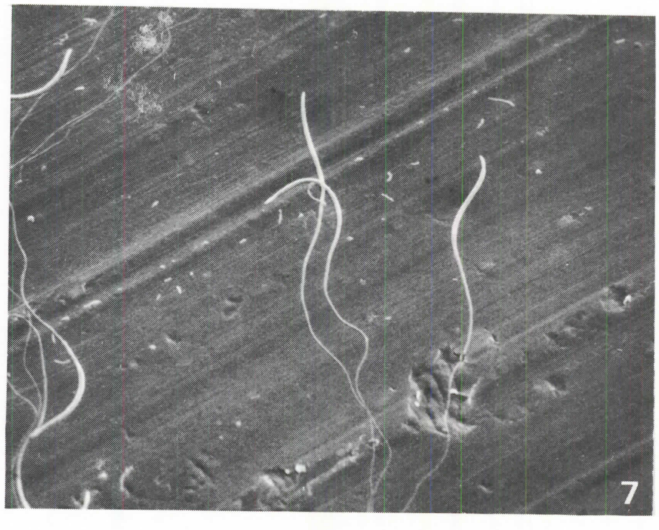

Fig. 7. Micrograph of fresh semen immediately after collection by scanning electron microscope (SEM). Normal sperm cells are seen in this micrograph. SEM X1,000.

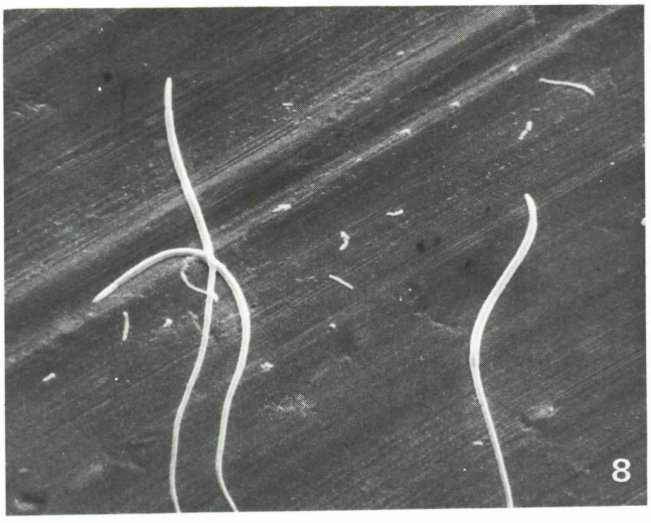

Fig. 8 The same as above. SEM X2,000.

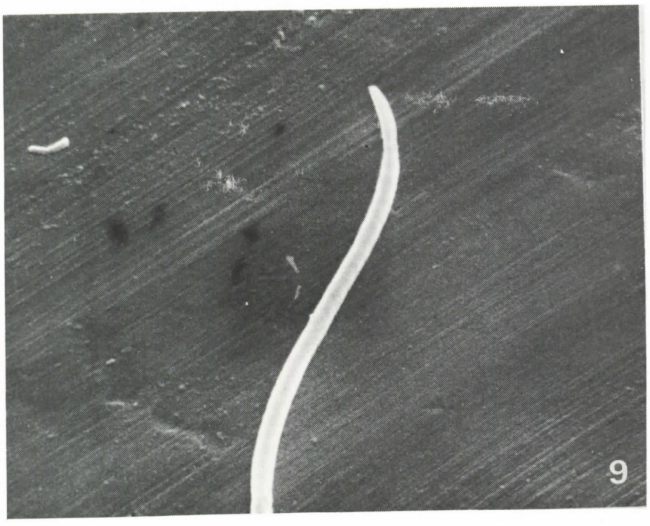

Fig. 9. A normal spermatozoon, the same as above. SEM X5,000.

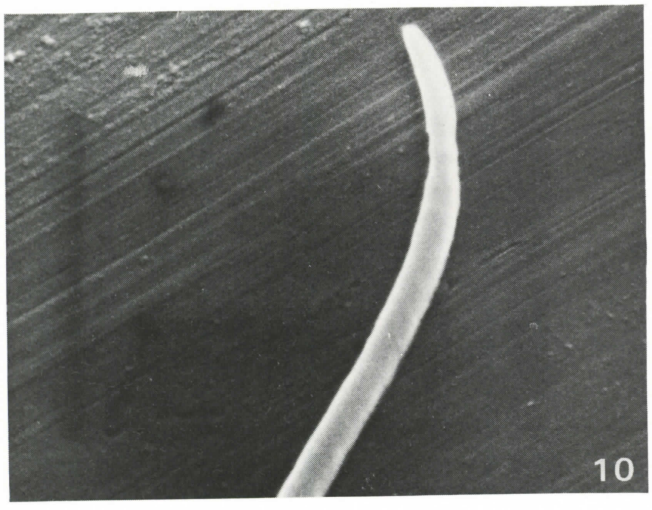

Fig. 10. The same as above. Acrosome is not isolated. SEM X10,000.

In these micrographs, the normal fowl spermatozoa with acrosome can be seen clearly.

SEM micrographs of the spermatozoa after thawing by the two freezing methods (pelleted freezing method and straw freezing method) and by the two diluents (5.7% glucose solution and 5.7% glucose solution 85 plus fresh egg yolk 15) are shown in Figs. 11 to 20.

The spermatozoa seen in these micrographs show the ultrastructural changes in acrosome (Figs. 11, 13, 14, 15, 16, 17, 18, 19 and 20), in the end of the sperm neck (Figs. 12, 16 and 18) and in the midpiece (Fig. 17). Thus, it becomes clear that even those spermatozoa that would be considered as normal in the sweep field of the light microscope, have suffered many serious ultrastructural changes when observed by a scanning electron microscope (SEM). HARRIS *et al.*[4] have shown that fowl spermatozoa are damaged ultrastructurally not only by freezing and thawing but also by the cryoprotectants used, e. g. glycerol and dimethylsulphoxide. MARQUEZ and OGASAWARA[5] also reported that the turkey spermatozoa are similary affected by the presence of glycerol.

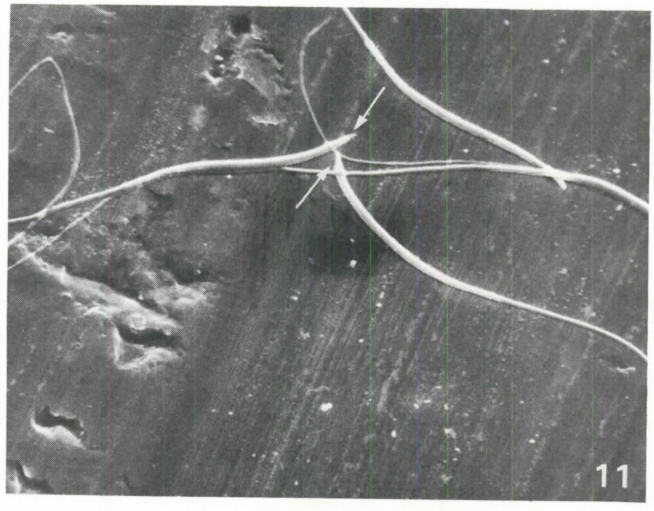

Fig. 11. Micrograph of spermatozoa diluted with 5.7% glucose solution after thawing by pelleted freezing method. Isolation of acrosome are seen in this micrograph. SEM X2,000.

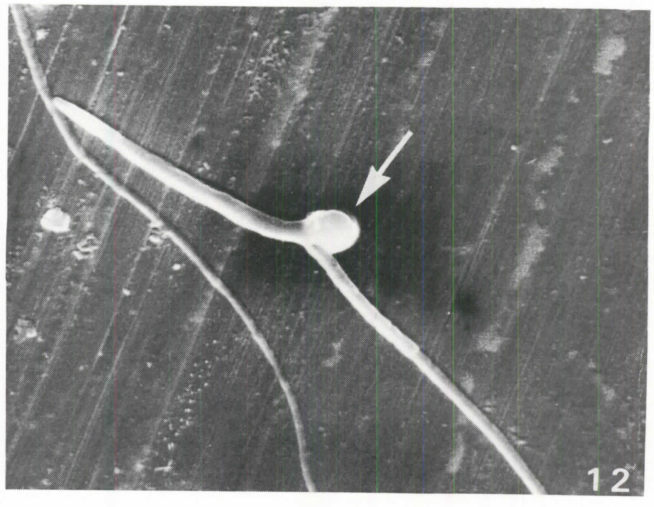

Fig. 12. The same as above. The swelling of the end of sperm head is seen. SEM X5,000

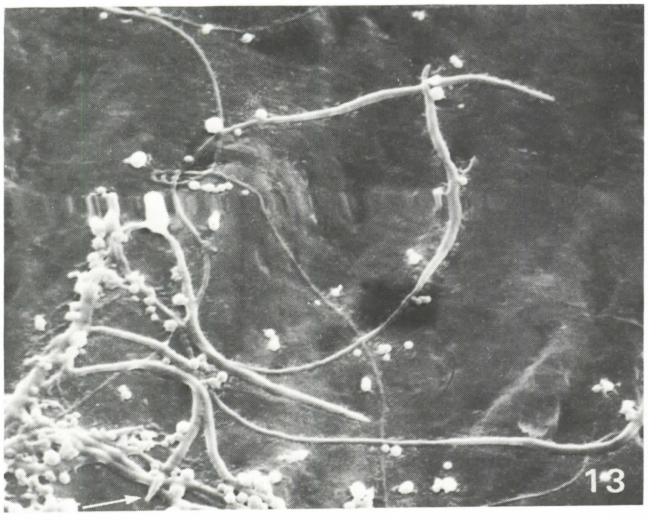

Fig. 13. Micrograph of spermatozoa diluted with 5.7% glucose solution plus fresh egg yolk after thawing by pelleted freezing method. Isolation of acrosome is seen in sperm cell. SEM X2,000.

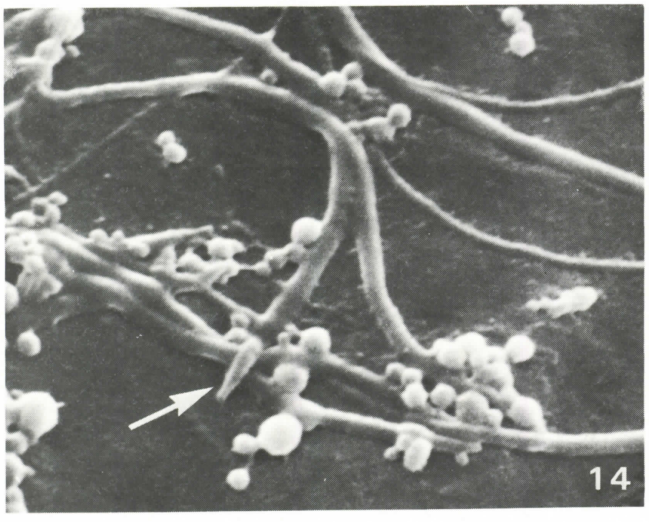

Fig. 14. The same as above. Enlargement of the isolation of acrosome in sperm cell. SEM X5,000.

Fig. 15. Micrograph of spermatozoa diluted with 5.7% glucose solution after thawing by straw freezing method. Isolation of acrosome is seen. SEM X2,000.

Fig. 16. The same as above. Isolation of acrosome and the swelling of the end of sperm head are seen in this micrograph. SEM X3,000.

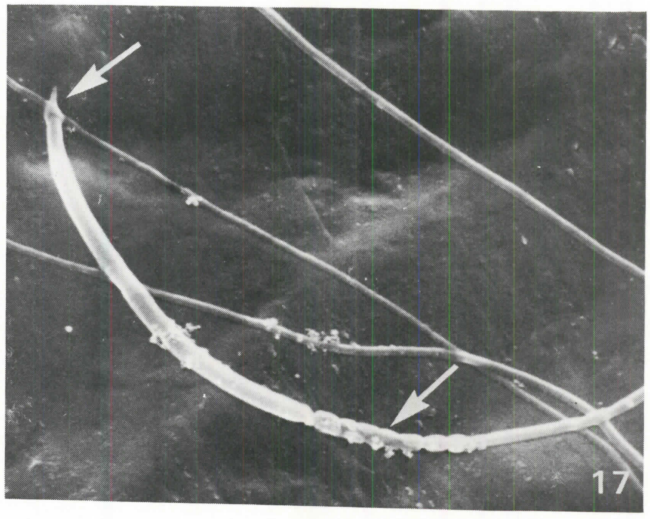

Fig. 17. The same as above. Isolation of acrosome and the abnormality of midpiece are seen in this micrograph. SEM X5,000.

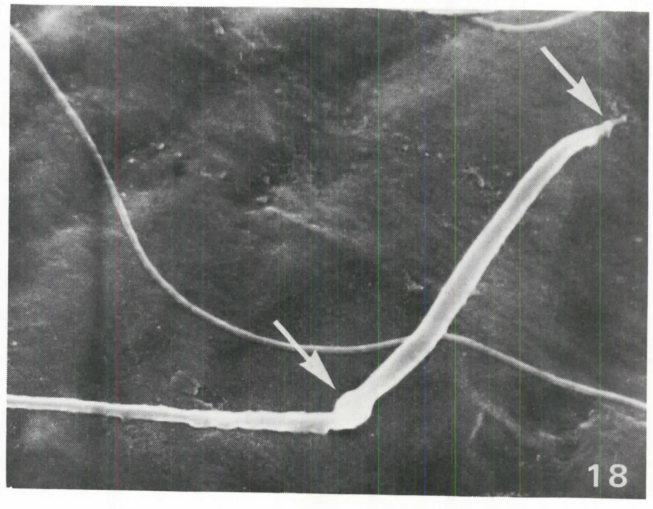

Fig. 18. The same as above. Isolation of acrosome and the swelling of the end of sperm head are seen in this micrograph. SEM X5,000.

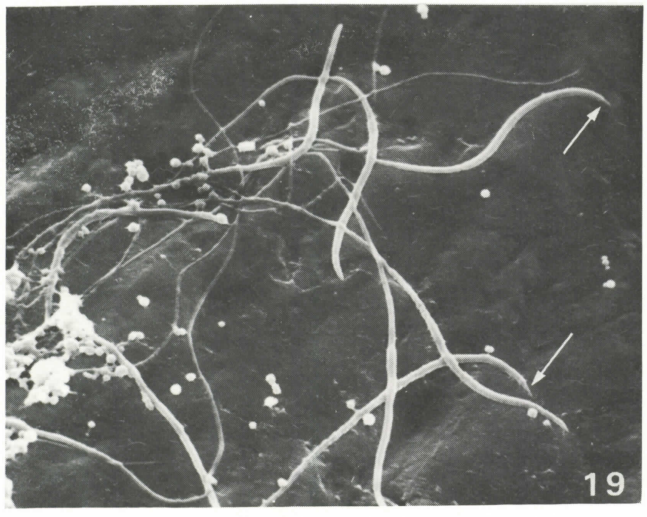

Fig. 19. Micrograph of spermatozoa diluted with 5.7% glucose solution plus fresh egg yolk after thawing by straw freezing method. Isolations of acrosome are seen in this micrograph. SEM X2,000.

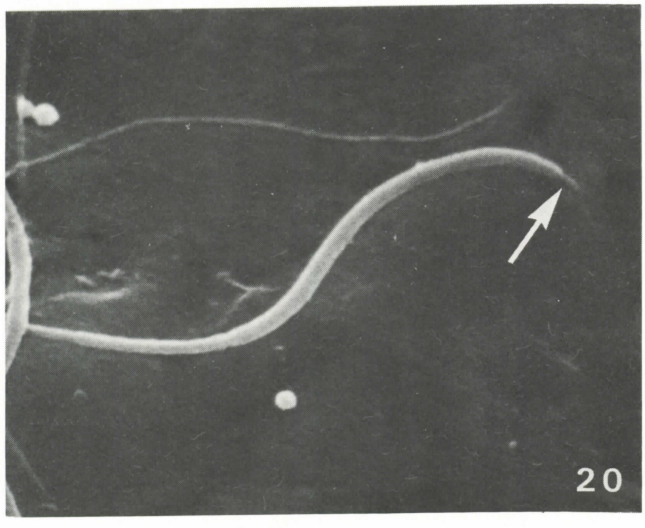

Fig. 20. Enlargement of Fig. 19. Isolation of acrosome in sperm cell is seen. SEM X5,000.

As mentioned above, in the present experiment, fairly morphological changes of neck-bending of the sperm cell in the midpiece were observed, as it was hitherto revealed by the light microscope.[11] Moreover the isolation of the acrosomal cap and the swelling at the end of the sperm head were observed in a new way. These ultrastructural changes in the sperm cell could not be observed by the usual light microscope possibly due to the freezing process. Perhaps, these morphological changes in the sperm cell may be responsible for the decrease in fertility.

SUMMARY

The purpose of the present study was to investigate the motility and the ultrastructure in fowl spermatozoa frozen by the pelleted and straw methods with scanning electron and the light microscopes. In either case of the pelleted and straw methods, the isolated

acrosome was observed in freeze-thaw fowl spermatozoa but the differences between the two methods were not clear. Also in the freeze-thaw fowl spermatozoa, the swelling at the end of the sperm head was observed. At the same time the neck-bending of the midpiece could be observed as clearly as in the case of the light microscope.

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鶏凍結精子の走査電子顕微鏡による研究

前田　照夫・寺田　隆登・渡辺　守之

最近走査電子顕微鏡による精子の形態学的研究が行なわれるようになり，哺乳類の精子は凍結保存により先体に著しい悪影響を被ることが知られている。一方家禽精子においても哺乳類の精子と同様に融解精子先体に著しい変化の認められることが報告されているが，凍結融解操作によってもたらされる一連の精子の損傷について観察した報告は少ない。

本研究は光学顕微鏡と走査電子顕微鏡を用い，錠剤化凍結法とストロー凍結法により，それぞれ二種類の希釈液を用いて凍結処理し，融解後の鶏精子の活力および畸形精子の微細構造について観察した。その結果，走査電子顕微鏡を用いれば，従来の光学顕微鏡下で通常認められる首曲り畸形精子の他に精子先体の離脱や，精子頭部末部の膨化が認められた。しかし凍結法，希釈液によるそれら畸形精子の出現割合の差異は明らかでなかった。