Reprod Nutr Dev (1994) 34, 427-437

Original article

Effect of acrosome defects and sperm chromatin decondensation on fertility and litter size in the rabbit. Preliminary electron-microscopic study

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(Received 2 May 1994; accepted 12 July 1994)

Summary — Sections of ejaculated spermatozoa from rabbits with known fertility were observed by transmission electron microscopy. Acrosome defects, odd head shapes, the presence of spermatids and the decondensation of sperm nuclei were recorded. The fertility negatively correlated with the number of acrosomal defects, while litter size was negatively related to nuclear decompaction. Sperm nuclear decondensation was present in all ejaculates. Its frequency was correlated with that of spermatids in ejaculates.

rabbit / sperm fertility / ultrastructure / acrosome / chromatin decondensation

Résumé — Effet des anomalies de l'acrosome et de la décondensation de la chromatine sur la fertilité et la taille des portées chez le lapin. Étude préliminaire des spermatozoïdes en microscopie électronique. *Des coupes de spermatozoïdes issus de lapins de fertilité connue ont été étudiées en microscopie électronique par transmission. Les défauts d'acrosomes, les anomalies morphologiques, la présence des spermatides et la décondensation spontanée des noyaux de spermatozoïdes ont été quantifiés. La fertilité est corrélée négativement au nombre d'acrosomes anormaux, tandis que la taille des portées l'est au nombre de noyaux décondensés. La décondensation spontanée de noyaux de spermatozoïdes est présente dans tous les éjaculats. Sa fréquence est corrélée à celle des spermatides dans l'éjaculat.*

lapin / fertilité / spermatozoïdes / ultrastructure / acrosome / décondensation de la chromatine

INTRODUCTION

Fertility is not generally considered as a problem in the rabbit. However, reproduction performances and sperm characteristics differ between breeds (Amann, 1966; Hulot and Matheron, 1981). These are genetically determined and males play an important role in fertility (Napier, 1961). The usual rabbit sperm characteristics, volume and concentration, have been described previously (Holtz and Foote, 1972) and while they are close to those found in the human (Williams *et al,* 1991), rabbit seminal plasma differs from most other mammals by the presence of numerous prostatic granules (Nicander *et al,* 1974). The usual sperm characteristics are sufficient to evaluate the potentialities of fresh semen in most cases, but they are not enough to explain differences between breeds. In this respect, the difference in litter size appears to be an important factor affecting reproduction in some breeds. Litter sizes are regularly diminished when frozen spermatozoa are used for insemination (Parrish and Foote, 1986), and freezing induces a slight nuclear decondensation (Courtens *et al,* 1989). For this reason, we have reexamined sperm ultrastructure in rabbit with special attention to nuclei. Sperm chromatin condensation is an important factor affecting male fertility (Kvist, 1983; Rodriguez *et al,* 1985; Huret, 1986). This is generally measured after drastic treatment, such as application of detergents (Kvist *et al,* 1987) or DNA and protein histochemistry and immunocytochemistry (Courtens *et al,* 1989, 1991).

In the present paper, we describe spontaneous decondensation of the chromatin in about 6% of spermatozoa in rabbit ejaculates without any experimental treatment or procedure other than preparation of the cells for histology. The observation of so many spontaneous sperm chromatin decondensations is new. It was not reported in previous ultrastructural studies on rabbit mature germ cells (Koehler, 1970; Fléchon, 1973; Courtens *et al,* 1976). Only Plöen (1972) described the decondensation of spermatid nuclei induced by experimental chryptorchidism. We have studied its occurrence in 50 bucks, and its significance in relation to fertility, litter size, and several parameters potentially affecting fertility, such as acrosome defects, the presence of spermatids and abnormally shaped nuclei, in 27 male rabbits with known fertility and prolificity.

MATERIALS AND METHODS

Animals and measurement of fertility

Crude fertility and mean litter sizes were recorded for 27 adult male rabbits used routinely for reproduction in 2 breeding centres. The crude fertility was defined as the percentage of pregnant females per mating for a 1-year period, and mean litter size was determined per successful mating for the same period. Since the number of matings (mean ± SD = 32.5 ± 25.2) was different for every buck, fertility and litter size were weighted by this parameter. Eleven males belonged to the so-called 'hyperfécond' A1029 strain, and 16 to the A1229 Chinchilla Rex strain, which will be hereafter referred to as the main strains.

Epididymal and/or ejaculated spermatozoa were also collected from 10 Californians, 4 New Zealand, 6 'Fauves de Bourgogne', and 2 lapin-lièvre from fertile animals with unrecorded data. They were used in pre-series qualitative experiments to test for the occurrence of the sperm defects described in the following sections.

Sampling

Sperm samples from animal with known fertility were obtained in March, June, October and December. One or 2 successive ejaculates (15-min interval) were obtained using artificial vaginas. These were immediately fixed with 5 vol 4% glutaraldehyde/PBS (phosphate–buffered saline) pH 7.4 for 24 h (4 h at room temperature and 20 h at 4°C). After centrifugation (2 000 *g*, 10 min) and

washing in PBS, they were post-fixed with 1% osmium tetroxide in PBS for 2 h at room temperature, and were embedded in diglycyl ether (formerly EPON 812) using routine procedure. Ultrathin sections were stained with uranyl acetate and lead citrate.

Analysis

At least 200 sperm heads were observed in each sample using transmission electron microscopy. The frequencies of 4 sperm defects were recorded. Abnormal acrosomes were classified as such when they were vesiculated (fig 1) or when they were empty (fig 2). The number of spermatids (fig 3), odd-shaped heads (figs 4 and 5), and decondensed nuclei (fig 6) was counted on microphotographs at a final magnification of 3 500. The effects of strain on fertility, litter size and the frequency of the 4 recorded defects were evaluated by analysis of variance. Correlations between fertility, litter size and sperm parameters were calculated with the strain as only fixed effect. From this analysis, residual correlations were calculated. The effect of the frequency of the 4 recorded sperm defects on fertility and litter size was analysed using the following multiple regression model: $X_{ij} = \mu + Si + b_1 Y_{1ij} + b_2 Y_{2ij} + b_3 Y_{3ij} + b_4 Y_{4ij} + E_{ij}$, where X_{ij} is fertility or litter size of the jth male from the strain i, and $b_1,...b_4$ are the partial regression coefficients on the 4 sperm defect frequencies $Y_{1ij},...Y_{4ij}$, respectively (Tomassone *et al*, 1983). The significance of each of these partial regression coefficients was verified by a Student's t test.

RESULTS

Sperm defects

No statistical difference was found between the sperm defects in the first and second ejaculates when both were available. No counting was performed in the epididymal sperm of the pre-series animals. There was a significant difference between the 2 main strains for the frequency of decondensed nuclei ($P < 0.05$). There were positive correlations between the frequencies of all the sperm defects, but only the correlations between abnormal shapes and spermatids or abnormal acrosomes were highly significant (table I).

Acrosomes

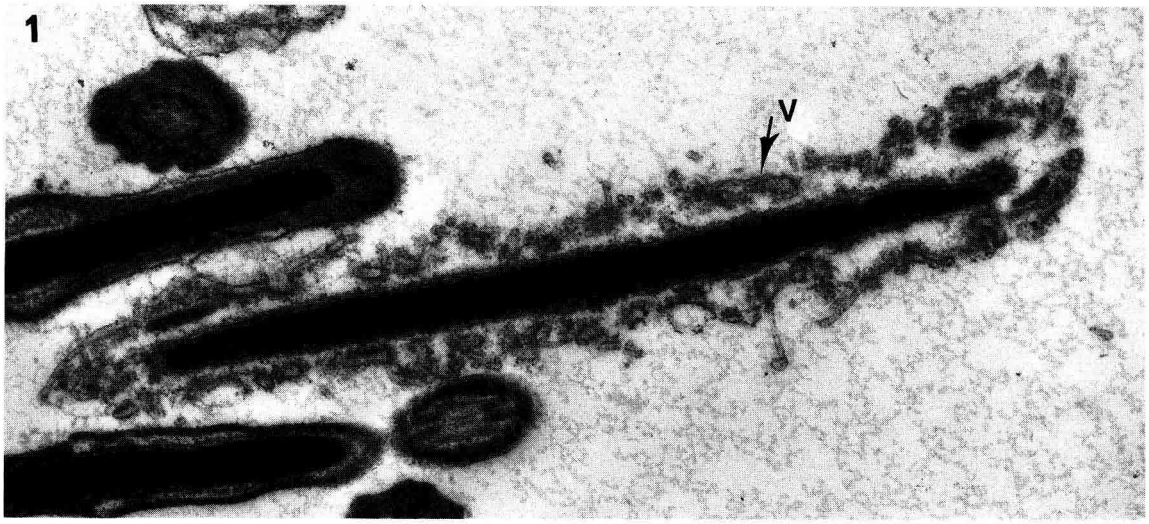
Acrosomal defects were rare in epididymal spermatozoa from the pre-series experiments. They were found in all ejaculates and could be divided into several types. Empty acrosomes were quite frequent (fig 2) and were generally associated with plasma membrane damage ranging from membrane breaking to a rearrangement similar to that found in acrosome reaction (fig 1). In a few cases, acrosomes were not spread on one side of the nuclei. Since most of these defects lead to acrosome destruction and the affected cells could not fertilise, they were not recorded separately.

Abnormally shaped spermatozoa

Abnormally shaped cells were found in the epididymis of the pre-series, and in all the ejaculates (figs 4 and 5).

Nuclear decondensation

Spontaneous nuclear decondensation occurred in all rabbit epididymal sperm and ejaculates. Different degrees could be observed from discrete separation of adjacent lamella of chromatin to almost total destruction of the sperm head (fig 6). Their occurrence in almost all ejaculates suggests that affected cells will normally be destroyed later. For this reason, they were classified as 'decondensed', whatever the degree of chromatin alteration and the eventual accompanying defects. Their mean number reported here only reflects cells, which were detected at the 3 500 x magnification used. Acrosome damage was not recorded for such cells even if it were present.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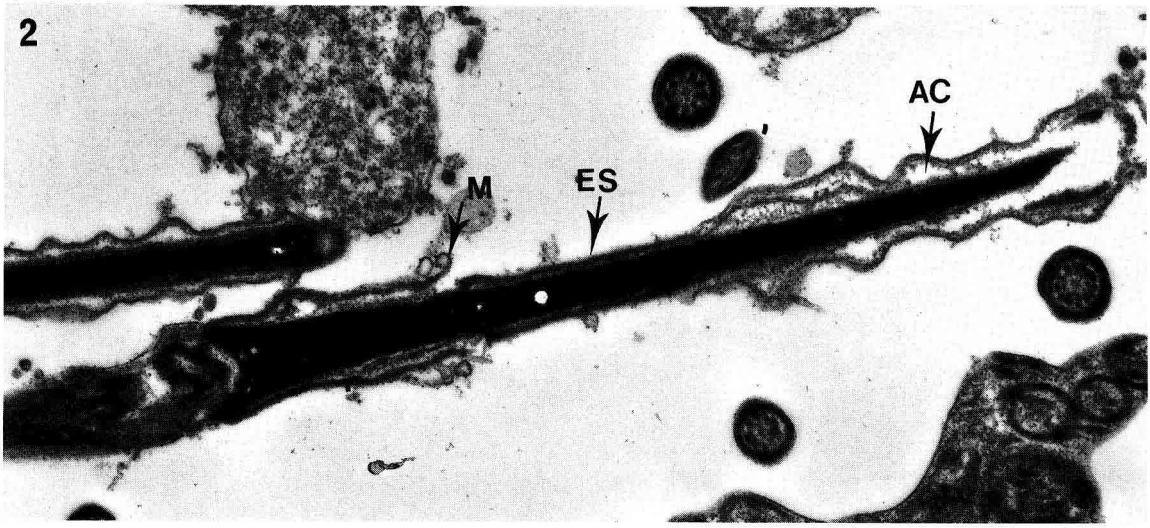
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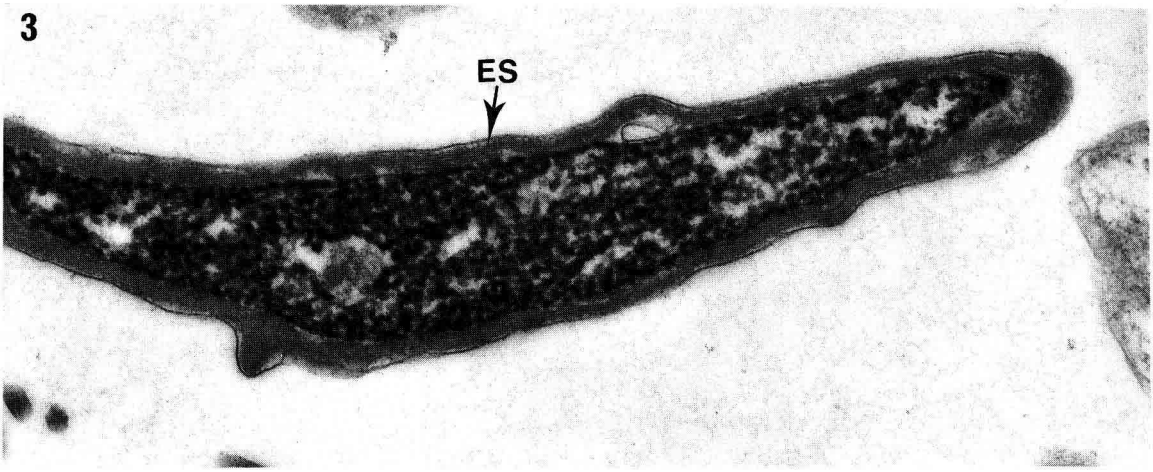
3
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Table I. Frequencies of 4 sperm defects in 27 males from 2 strains.

	Abnormal acrosomes (%)	*Abnormal shapes (%)*	*Decondensed nuclei (%)*	*Spermatids (%)*
Population mean ± SD	11.8 ± 11.4	2.6 ± 2.7	5.8 ± 4.0	2.9 ± 4.1
Population min – max	1–46	0–13	0–15	0–20
Mean for hyperfécond ± SD (N = 11)	15.6 ± 15.5	1.8 ± 2.0	3.8 ± 2.1	1.8 ± 3.1
Hyperfertile min – max	1–46	1–7	0–7	0–10
Mean for Rex ± SD (N = 16)	9.2 ± 6.8	3.2 ± 3.0	7.3 ± 4.4	3.7 ± 4.7
Rex min – max	11–22	0–13	2–15	0–20
Difference between breeds	ns	ns	*	ns
Correlation with				
Abnormal shapes	0.52 **			
Decondensed nuclei	0.34 ns	0.35 ns		
Spermatids	0.27 ns	0.66 **	0.43 *	

ns: correlation not significantly different from zero; * $P < 0.05$; ** $P < 0.01$.

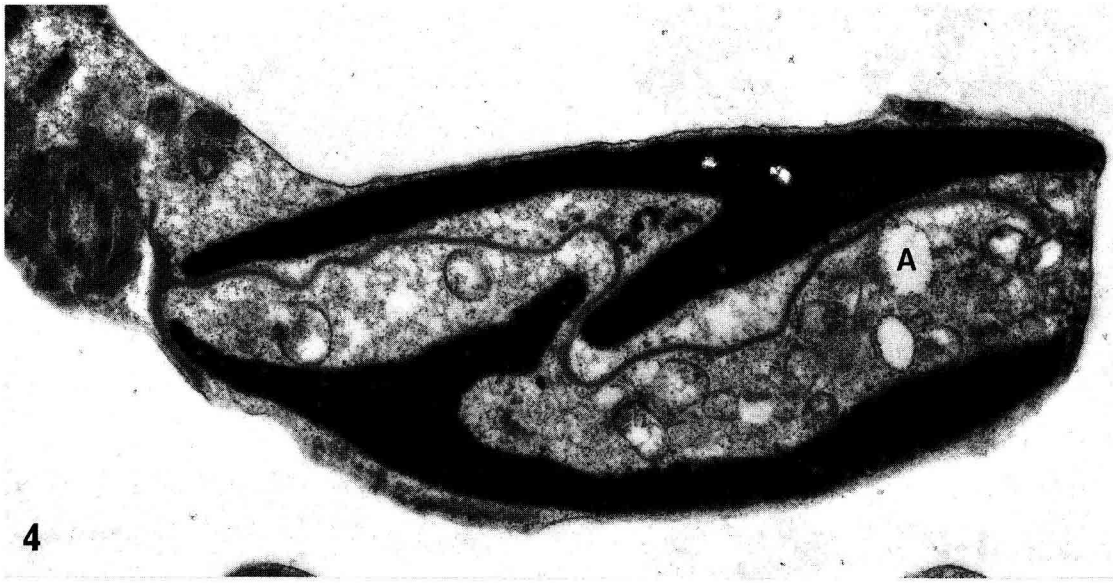
Spermatids

Few spermatocytes and all types of spermatids, from early stage-1 spermatids to elongating and fully elongated spermatids, could be found in low numbers in ejaculates (fig 3). No ejaculate contained all the various types of spermatids, but stages 1–3 (young, round spermatids) and 11–14 (elongating to fully elongated spermatids) were frequent. Round germ cells were numerous in several rabbits and some fully elongated spermatids often displayed differences in nuclear and cytoplasm maturity. The chromatin was often typical of stage-11 spermatids, while the acrosome was fully differentiated as in stage-14 spermatids (fig 3).

Relationship to fertility and litter size

The fertility, litter size and the 4 parameters studied in all rabbits are given in tables I and II. Quite high numbers of abnormal acrosomes were found in most ejaculates (mean 12%) but these did not differ significantly between breeds. The abnormal shapes and the presence of spermatids were also similar between breeds. No relationship was noted between the presence of spermatids and the season or high temperatures during the months beforehand (see Rathore, 1970). Decondensed nuclei were more numerous in Rex than in hyperfécond rabbits (7.3 *vs* 3.8%, $P < 0.05$).

Fig 1. Section through the anterior part of vesiculised acrosome. The plasma membrane and the membrane of the acrosome have fused to form vesicles (V); x 26 000.
Fig 2. The anterior part of the acrosome is empty (AC) while the equatorial segment of the acrosome (ES) is intact. The plasma membrane (M) is ruptured; x 19 000.
Fig 3. The chromatin appearance is that of a stage-11 elongating spermatid, while the equatorial segment of the acrosome (ES) is fully formed (this occurs at stage 14 of spermiogenesis). Such a picture can be obtained either from a spermatid with a desynchronization between differentiations of the nucleus and the cytoplasm, or from a spermatozoa with a decondensed nucleus; x 24 000.



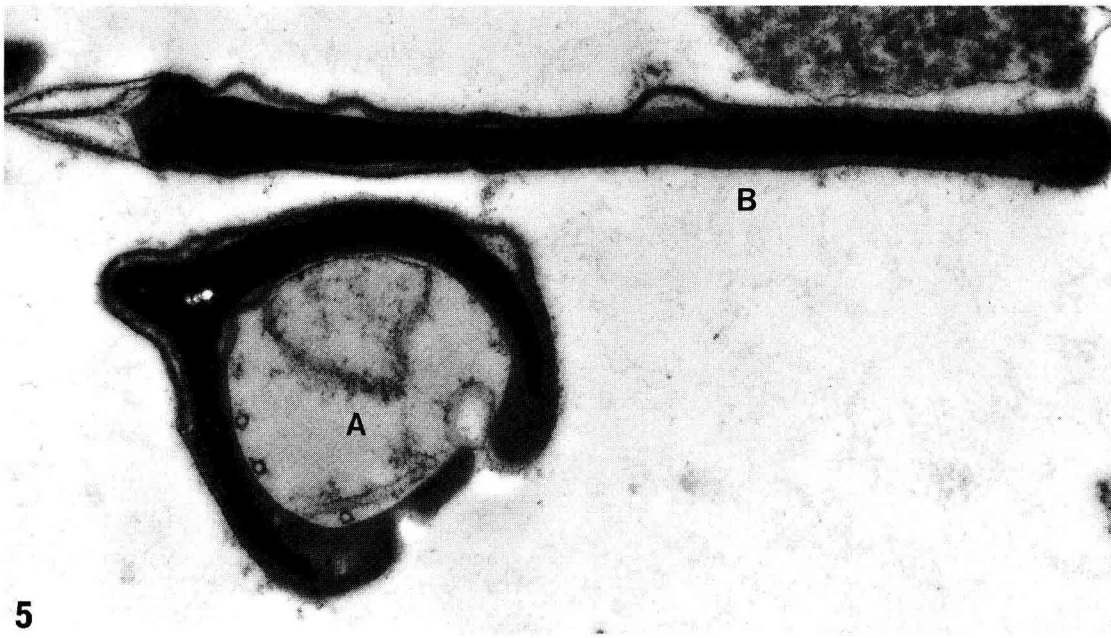


Fig 4. Sections through 2 odd-shaped nuclei located in one cytoplasm; x = 25 000.
Fig 5. Abnormal sperm head (A) compared with a normal head (B); x 19 000.

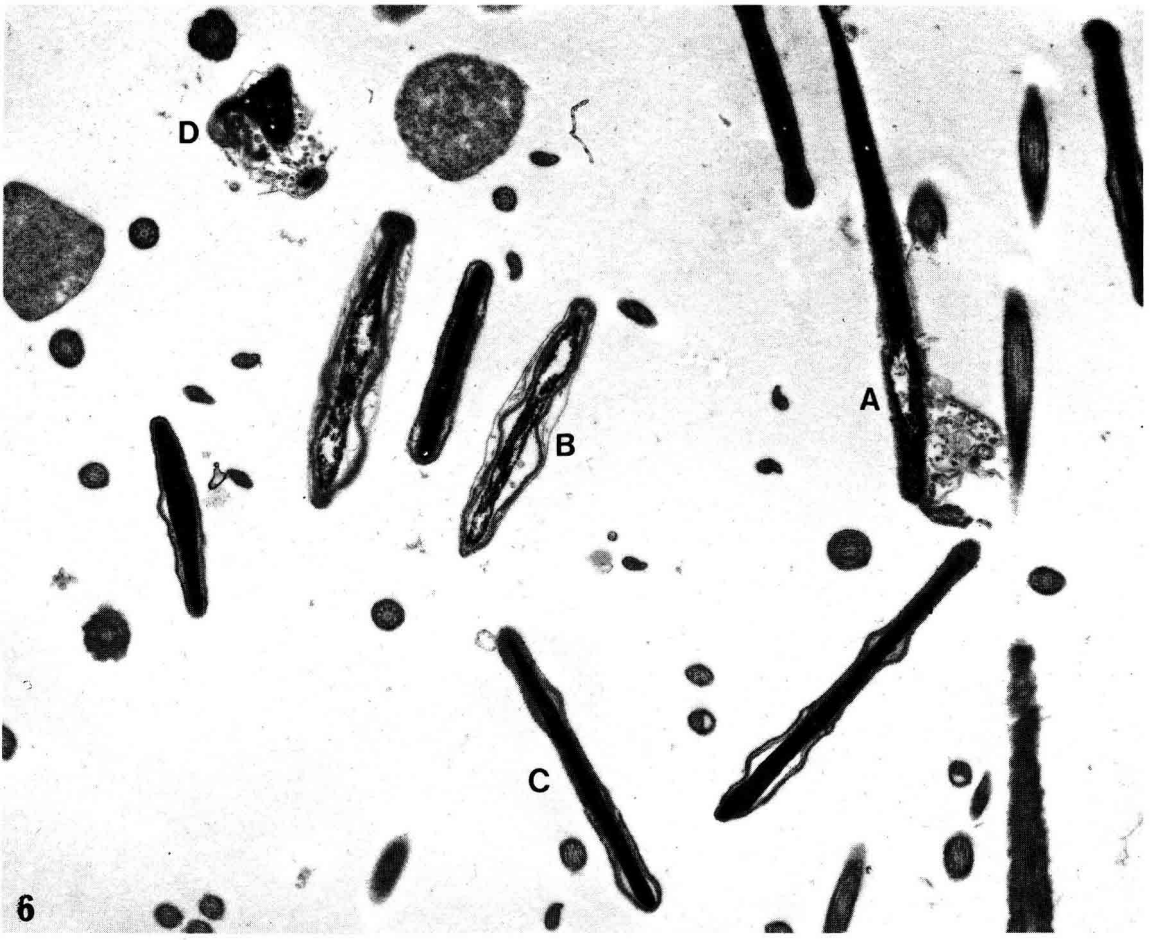


Fig 6. The posterior part of the sperm nucleus in cell A is decondensed. No plasma membrane or acrosome is present. Cell B is also decondensed to a larger extent. The acrosome and plasma membrane are intact. This can be compared with cells C (normal spermatozoon) and D (abnormally shaped spermatozoon); x 9 000.

Fertility

There were no significant differences between strains (75 *vs* 79%). There was a significant negative correlation between fertility and frequency of acrosome defects ($r = -0.55$, $P < 0.01$). The correlations with other sperm defects were not significant. This was confirmed by the regression of fertility on sperm defects, which was only significantly negative on abnormal acrosomes frequency ($b = -0.9 \pm 0.28$).

Litter size

Litter size was significantly lower in Rex than in hyperfécond does (5.7 *vs* 7.3%, $P <$

Table II. Fertility and litter size, mean ± standard deviation, correlation (r) and weighted multiple regressions with strain effect (b) on 4 sperm defects.

	Fertility (%)	*Litter size*
Hyperfécond + Rex mean ± SD	77.3 ± 19.9	6.3 ± 1.7
Hyperfécond mean ± SD	74.9 ± 5.3	7.3 ± 0.3
Rex mean ± SD	78.8 ± 4.4	5.7 ± 0.2
Difference between breeds	ns	**
Correlation *(r)*		
Abnormal acrosomes (%)	−0.55 **	−0.01 ns
Abnormal shapes (%)	−0.33 ns	+ 0.46 *
Decondensed nuclei (%)	−0.15 ns	−0.26 ns
Spermatids (%)	−0.23 ns	+ 0.45 *
Weighted multiple regression with strain effect (b)		
Abnormal acrosomes (%)	−0.9 ± 0.28 **	−0.04 ± 0.01 *
Abnormal shapes (%)	−1.0 ± 1.7 ns	+0.12 ± 0.08 ns
Decondensed nuclei (%)	+1.2 ± 0.9 ns	−0.14 ± 0.04 **
Spermatids (%)	−0.30 ± 1.1 ns	+0.18 ± 0.05 **

ns: regression or correlation not significantly different from zero; * $P < 0.05$; ** $P < 0.01$.

0.001). The multiple regression of litter size on sperm defects was significantly negative for the frequency of decondensed nuclei ($b = -0.14 \pm 0.04$) and to a lesser extent for abnormal acrosomes ($b = -0.04 \pm 0.01$). It positively correlated with the presence of spermatids.

DISCUSSION

In this work, we have focused our attention on 4 possible semen parameters that may influence rabbit fertility. Among them, acrosomal defects had a deleterious effect on fertility, and nuclear decondensation frequency was a negative factor for litter size.

Acrosome function is necessary at the time of fertilisation to help spermatozoa passing the egg vestments using the so-called acrosome reaction (Yanagimachi, 1988). Severe intrinsic acrosome abnormalities (Holstein *et al,* 1973; Baccetti *et al,* 1991) and/or externally driven acrosome reactions (Agrawal and Vanha-Perttula, 1987; Tesarik and Mendoza, 1993) can dramatically affect the rate of fertilisation. The general features of the acrosomes of placental mammals are fragility (Sistina *et al,* 1993) and sensitivity to environmental factors, such as secretions of the male accessory glands (Courtens *et al,* 1984) or divalent cations (Yanagimachi, 1988). Acrosomal defects in rabbits are not fundamentally different from other species (Jones, 1973; Yanagimachi, 1975) and therefore it is probable that most of them are induced by factors present in seminal plasma. This is further accentuated by the fact that few acrosomal abnormalities were found in epididymal spermatozoa. To know the time of their appearance will need further characterisation, especially in conjunction with the presence of prostatic true apocrine secretions in rabbit semen (Nicander *et al,* 1974). They should contain lytic enzymes excreted

with cell debris. However, the origin of several of the defects, such as abnormal spreading of the acrosomal cap on the head, is obviously related to spermiogenesis. The number of acrosomal defects is also positively correlated to the number of abnormal cells produced in the testis, a factor that was recently shown to affect fertilisation in mutant mice (Meistrich *et al,* 1994). The spermatozoa with defective acrosomes are probably unable to fertilise. However, their high frequencies should be related to enhanced fragility, known to affect fertilisation. Litter size could also be impaired if too many cells are affected (see table II).

The presence of spermatids and the frequency of abnormally shaped spermatozoa in ejaculates has no effect on fertility. The fact that all the types of spermatids and several spermatocytes could be found in ejaculates indicates that some seminiferous tubules could have been severely impaired, since spermatids penetrate deeply into the seminiferous epithelium during their differentiation. The presence of spermatids in ejaculates is probably not of severe pathological origin since most rabbits in this study were reported as healthy. This observation is rare in most mammals and has mostly been reported in humans insofar as many cells in human ejaculates look like old spermatids and also contain spermatid proteins (Gatewood *et al,* 1990). The positive correlation between litter size and the number of spermatids in ejaculates cannot be seriously attributed to the fertilisation of oocytes by spermatids. It is most likely due to the fact that frequencies of spermatids and decondensed nuclei are correlated.

The premature decondensation of several types of spermatid nuclei can be experimentally induced in the rabbit by cryptorchidism (Plöen, 1972). This is present in most ejaculates as well as in the epididymal sperm studied in the pre-series. This new observation indicates that: 1) spontaneous decondensation of male germ nucleus is not restricted to the testis, one breed or one breeding place; 2) the decondensation is not necessarily of pathological origin since it is present in almost all animals, but the positive correlation with the frequency of spermatids indicates a probable testicular origin; 3) the different locations and degrees in decondensation that we observed reveal that the phenomenon is not an explosion, which could affect all cells sensitive to a common starter, but seems rather to affect cells at random or at the point when they reach a particular maturity; and 4) no differences according to season or previous high temperatures, which are known to affect rabbit germ cells (Rathore, 1970) were noted.

The classical factors known to affect sperm chromatin condensation are divalent cations present in male accessory gland secretions (Kvist *et al,* 1987) and reduction of disulfide bridges in protamine (Perreault *et al,* 1984, 1987). These can probably be excluded as an explanation for spontaneous decondensation in the epididymis when disulfide bridges are actively formed in sperm nuclei (Bedford and Calvin, 1974) and before the cells are mixed with accessory gland secretions. The biochemical composition of nuclei should also be considered, since rabbit sperms have only one protamine which differs from other mammalian protamines 1 by the *N*-terminal amino-acid sequence (Ammer and Henschen, 1988). However, no exotic association of this special protamine with the DNA is established, and the super-organisation of rabbit sperm chromatin in lamella (Koehler, 1970; Fléchon, 1973) is not fundamentally different from that found in several other mammalian species (Courtens *et al,* 1991a). The presence of nuclear sperm proteinases, involved in sperm nuclear decondensation (Perreault and Zirkin, 1982), or the possible phosphorylation of protamine (Pirhonen *et al,* 1993) have not yet been demonstrated in rabbit.

The fact that chromatin decondensation is observed from epididymis to ejaculate and is correlated to the number of spermatids is a good indication for a defect induced in the testis. The observation of different steps in sperm nuclear decondensation, from slight to severe, suggests that they normally lead to cell destruction. Moreover, the number of decondensed nuclei reported here might be largely under-evaluated: some spermatozoa were probably destroyed before the observation, and others were not given their chance to decondense, due to fixation. Because sperm that is decondensed to a visible exent in ejaculate will probably soon be disrupted, it has little if any chance of fertilising. However, a high ability to decondense for a fertile spermatozoon or the fertilisation by a poorly condensed or a pre-decondensed cell could explain the negative effect decondensation plays on litter size, probably *via* embryonic death.

The observation that the 2 main strains differ significantly in both litter size and the frequency of sperm nuclear decondensation is a new result. This can be considered as a preliminary, since the number of ejaculates was necessarily limited, due to the heaviness of the quantitative electron microscopy. Correlations are also at the limit of significance when a strain effect is introduced as a covariate, but are significant when simple correlation is used (not shown). However, the occurrence of such a defect is now established in about 50 animals and should be considered as a main parameter affecting reproduction performance in the rabbit. The slight positive correlation of abnormally shaped spermatozoa on litter size is probably a side effect. This relation only appeared when strains were introduced as covariates for calculation, and was not significant when data from each strain were considered separately. In both cases, it was not confirmed by multiple regression analysis.

CONCLUSION

The present study indicates that, among the studied sperm abnormalities, acrosome defects are the main factors affecting fertility, indicating that increased acrosome fragility could be a source of further fertilisation problems. Poor nuclear condensation or precocious nuclear decondensation is related to low litter size, is enhanced in some genotypes, and hypothetically induces embryo mortality. Most of these defects could be generated in the testis. X-ray spectrophotometric analysis of sperm nuclei elements in the same rabbits (Ekwall *et al,* submitted for publication), and the study of some sperm oligonucleoproteins (results not shown) support this last conclusion.

ACKNOWLEDGMENTS

This work was solicited and funded by the board of INRA, AIP 'regulation intragonadique'. We wish to thank G Thébault and H de Rochambeau for providing computer records on fertility and reproduction parameters, and A Beguéy for photographic work.

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