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Meiosis in fetal freemartin gonads and in rat fetal ovaries *in vitro*

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Summary. In the gonads of male or female calf fetuses and of fetal freemartins, the germ cells may present nuclear aspects resembling those described in other species as the « preleptotene chromosome condensation stage ». The maximum of these aspects occurred between 60 and 80 days post-insemination. The fate of the cells presenting these aspects is not know, and it is not certain that this nuclear stage really is part of meiosis.

If one takes as a criterion the more conventionally recognized figures (leptotene, zygotene), meiosis was seen in the three freemartin fetuses aged 77 to 88 days, but the percentage of meiotic cells was low.

In fetuses older than 90 days no meiotic cells were present in those gonads in which seminiferous cords developed. In gonads which contained no seminiferous cords, meiotic figures were seen only in three animals under 133 days of age ; in older animals meiosis was not present.

The presence of seminiferous cords in the gonad is incompatible with the presence of meiotic germ cells. In every case, the germ cells disappeared from the freemartin gonad after day 186, except for a few which persisted until day 222 because they were located in seminiferous cords.

In an attempt to determine whether meiosis could be influenced by various agents, rat fetal ovaries were cultivated *in vitro* with or without estradiol, an anti-estrogen, progesterone, methyladenine, ram rete fluid or « inhibine » extracted from ram rete fluid. In these preliminary non-quantitative experiments, no definite alterations of meiosis were noted.

The biology of the germ cells raises several fundamental questions in biology. The conditions permitting them to multiply, differentiate and grow in the gonads remain unknown since isolated embryonic germ cells have not yet been cultivated *in vitro*. The reason why XX germ cells never were seen to survive in a mammalian testicular gonad has also escaped analysis so far. The major characteristic of the germ cells is meiosis. Why should a germ cell undergo meiosis rather than mitosis at a certain stage of its history, and how is the timing of the process controlled ? These questions are of foremost importance in the biology of reproduction.

Freemartins in cattle provide one example of animals whose presumptive ovaries contain XX germ cells which disappear during prenatal development. The aim of the present work was to determine when the germ cells disappeared in these gonads and

whether they entered meiosis or not. Some preliminary concomitant experiments concerning studies on meiosis in fetal rat ovaries grown *in vitro* will also be briefly presented.

Material and methods.

The present study concerning meiosis in the gonads of fetal freemartins was initiated by one of us (J.P.), taking advantage of the series of freemartins that we collected during the last years (Jost *et al.*, 1972 ; Jost *et al.*, 1973*a*, *b* ; Jost *et al.*, 1975). The animals had been fixed in Bouin's fluid and serially sectioned at a thickness of 5 or 7μm. Staining methods included Haemalun-erythrosin, Tuchmann-Duplessis' trichrome, and Masson's trichrome.

The number of germ cells present in some of these gonads, and counted according to the Chalkley method, were reported earlier (Jost *et al.*, 1973*b*).

The condition of germ cell meiosis was carefully studied in every third section of the serial sections of the gonads (in a few fetuses only one gonad was available).

The determination of the meiotic stages of the germ cells in histological sections of the gonads is sometimes difficult or uncertain, as discussed earlier by Beaumont and Mandl (1962) for rats or Erickson (1966) for calf fetuses.

Approximative quantitative evaluations of the number of meiotic germ cells per cent germ cells were made in three median sections of the gonads, 250 mμ apart, by counting all the germ cells and identifying them as to meiotic stage. The number of cells counted varied from 910 in the younger animals to 0 in the older ones.

The methods for cultivating rat fetal ovaries *in vitro* were described earlier (Rivelis *et al.*, 1976).

Results.

Part one : Meiosis in fetal freemartin gonads

A) « *Preleptotene condensation* » *stage in calf gonads.*

Stahl, Luciani and their coworkers insisted on a transient chromosome condensation stage seen during oogenis in human (Stahl and Luciani, 1971), rabbit (Devictor

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PLATE 1

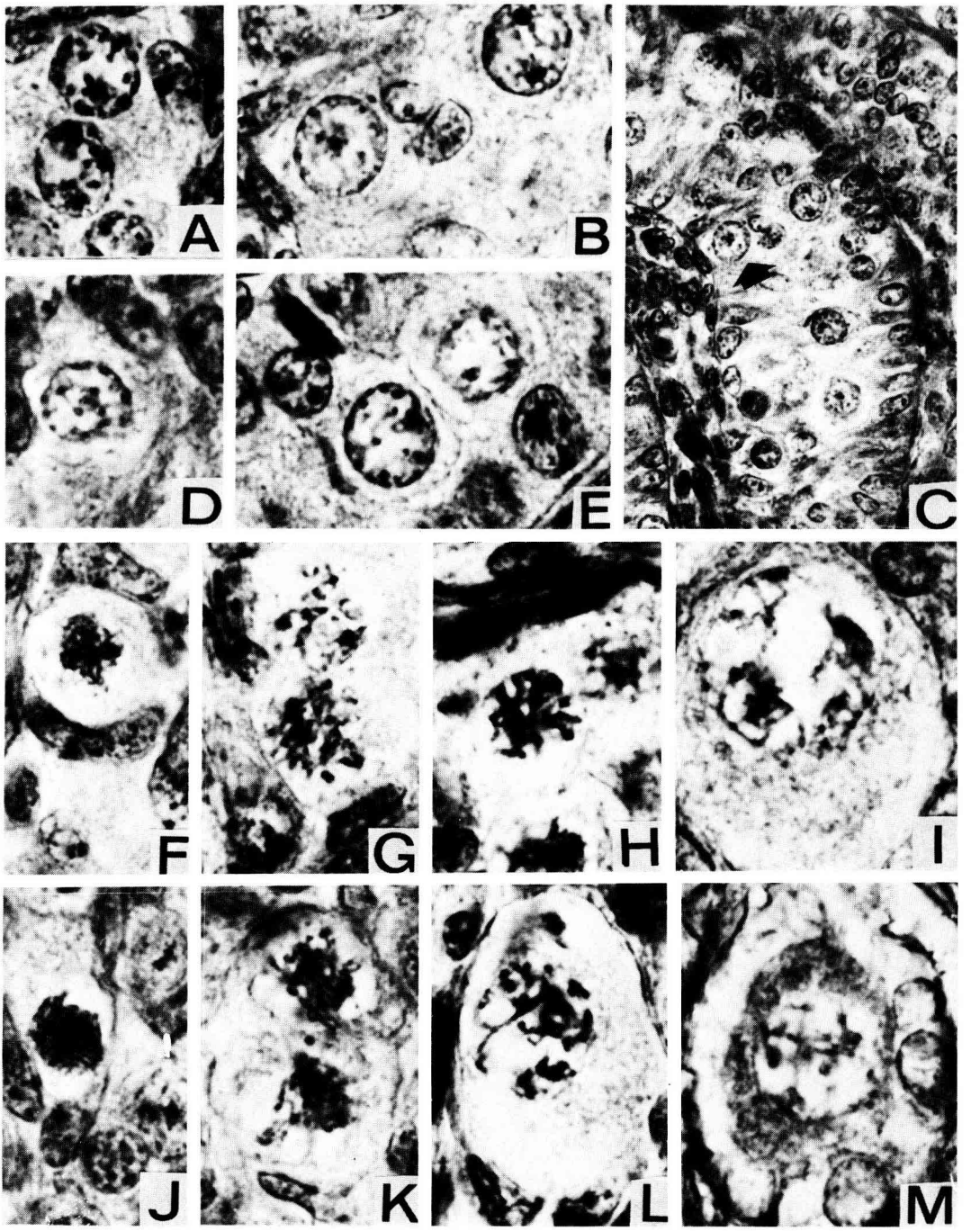
Varying aspects of germ cells in histological sections of normal calf fetuses and of freemartins.

A to E : « Preleptotene condensation stage » in a normal 70-day old female fetus (A), in a normal 89-day old male fetus (B, C shows the same germ cells in a seminiferous cord, see arrow, as well as other germ cells) and in two freemartins respectively 125 (D) and 97 days old (E).

F to I : Meiotic stages in normal females, namely, leptotene (F) and zygotene on day 70 (G), pachytene on day 89 (H), and dictyate on day 110 (I).

J to M : Meiotic stages in freemartins : leptotene (J) and zygotene on day 77 (K), probably degenerating pachytene on day 146 (L) and dictyate on day 133 (M).

Enlargement $\times$ 1 500, except for C ($\times$ 600).


A
B
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et al., 1973), sheep (Mauléon *et al.*, 1976) and mouse (Hartung and Stahl, 1977) presumptive ovaries. The transient condensation of the chromosomes resulted in masses which have been evaluated to be as numerous as the number of chromosomes. Decondensation preceded the true leptotene phase.

Preleptotene chromosome condensation has also been described in the human fetal testis (Luciani *et al.*, 1977) ; but, contrary to what occurs in the ovary, in the testis no decondensation and no meiosis ensue. The fate of the spermatogonia showing preleptotene condensation is not known, but it is « probable that a process of reversion to a mitotic behaviour takes place » (Luciani *et al.*, 1977).

Aspects resembling the stage of preleptotene condensation could be seen in some germ cells of calf fetuses of both sexes (plate I, A to E) already on day 36, before the gonad could histologically be recognized either as a testis or as an ovary (the fetuses were sexed with the chromosome studies of one of us, B.V.). We could not verify whether the number of the chromatin masses was equal to the number of chromosomes, because their aspect and shape were variable in the sections.

The number of oogonia showing this chromatine condensation increased in the normal ovaries, but from day 69 onward the definite meiotic phases could be recognized (plate I, F to I). In females meiosis seemed to occur during a period of approximately 4 months, since the first leptotenes are seen on day 69 and the last oogonia on day 185. These oogonia probably did not enter meiosis since no leptotene or zygotene was seen after this stage.

In male fetuses the proportion of germ cells presenting aspects similar to the « preleptotene chromosome condensation » (plate I, B and C) increased strikingly between days 60 and 80, when it seemed to reach a maximum. This stage approximately corresponds the time when meiosis occurs in females.

The number of germ cells involved was so high that it appears unlikely that all these cells degenerated. According to Matschke and Erickson (1969), gonocyte necrosis (first noted in testes aged 110 days) increases thereafter but « is not a prominent feature of the prenatal bovine testis ».

Since similar preleptotene condensation occurred both in males and in females, and since in males it was not followed by meiosis, the preleptotene condensation stage was not included in the meiotic stages and was not reckoned in the following results.

B) *Meiosis in freemartins.*

It should first be recalled that the development of the gonads of presumptive freemartin fetuses can be divided into three successive phases : 1) until day 50 or 52 the gonads resemble those of normal females ; 2) between day 52 and day 90 approximately the gonads cease growing and therefore look stunted in comparison with control gonads ; the number of germ cells does not increase ; 3) after day 90 the gonads resume growth to some extent and seminiferous cords, resembling those present in fetal testes, may appear (Jost *et al.*, 1973*a*, *b*).

1) *Freemartin fetuses under 90 days.* — In normal females the first leptotenes were observed in the deepest germ cells of the 69-day old control gonads (table 1). Thereafter the number of germ cells passing successively through the leptotene, zygotene and pachytene phases increased ; the changes proceeded from the deeply located germ

cells towards the more superficial ones. On day 90 the proportion of the germ cells which already had entered the meiotic prophase was evaluated to be 80 p. 100. Approximately one-fifth of the germ cells still were at the oogonia stage.

TABLE 1

Meiosis in freemartins and controls

Females		Freemartins			Males	
Age	meiotic stage	Age	meiotic stage		Age	meiotic stage
36	Preleptotene condensation	36	Preleptotene condensation		36	Preleptotene condensation
		39	*id.*			
					40	First seminif. cords
69	First leptotenes (L)	70	No meiosis		70	Preleptotene condensation
74	LZ (± 20 p. 100)					
77	LZ (± 30 p. 100)	77	LZ (< 4 p. 100)			
					80	Maximum prelept. cond.
		84	LZ (< 6 p. 100)			
89	LZPD (± 80 p. 100)	88	LZ (< 8 p. 100)			
		No seminif. cords		Seminif. cords		
		93	LZ (11 p. 100)	93	No meiosis	
				97	—	
		108	LZP (< 12 p. 100)			
		109	No meiosis	109	—	
110	LZPD (± 95 p. 100)	110	—	115	—	
		122	—	125	—	
		133	LZPD (< 12 p. 100)			
					130	Preleptotene condensation
		148	No meiosis	146	—	
		150	—	150	—	
		155	—			
		162	—	167	—	
		170	—	169	—	
185	PD + last oogonia	188	—	186	—	
		No germ cell left				
				222	—	
				No germ cell left		

Every third section was studied in the serial sections.
Percentages determined in three median cross-sections 250 mμ apart.
L = leptotene, Z = zygotene, P = pachytene, D = dictyate.

In three freemartins aged 77 to 88 days, leptotene, and zygotene stages were seen (Plate I, J and K) ; meiosis had not begun in two 70-day old freemartins (table 1). If compared with control females this series — though limited in number — would suggest that in freemartins : 1) the total number of germ cells is low ; 2) meiosis might

start somewhat later than in controls ; 3) a smaller percentage of the germ cells shows meiotic changes ; 4) the meiotic changes do not proceed beyond the zygotene phase during that period of development, whereas in the controls it passes the pachytene phase.

2) *Freemartin fetuses over 90 days.* — In control females the bulk of germ cells degenerate between days 110 and 190 approximately (Erickson, 1966). Most of the surviving germ cells have entered the meiotic prophase at the end ot this period. The few oogonia still present can be suspected to degenerate since no leptotene nor zygotene stage are seen after that age (an observation also made by Erickson, 1966).

The gonads of the freemartins aged more than 90 days may or not contain seminiferous cords (Jost *et al.*, 1973*a*, *b*).

In the 11 freemartins whose gonads contained seminiferous cords, many germ cells were located in these cords ; not one single meiotic cell could be found (table 1).

In the 12 animals whose gonads contained no seminiferous cords, meiotic stages (leptotene, zygotene and exceptionally pachytene) were found in the two youngest animals (93 and 108 days of age) ; in another one 3 weeks older, the percentage of meiotic cells was very low in comparison with normal control females. In the nine other animals, especially in all those over 133 days of age, no meiotic cells were present. It appears that the presence of seminiferous cords in the gonad prevents meiosis. Without any exception germ cells enclosed in seminiferous tubules never were meiotic. As a rule the germ cells located outside the cords did not enter meiosis either, with two apparent exceptions reported in table 2. One single probably degenerating pachytene cell (plate I, L) was found besides many oogonia in a 146-day old gonad in which one single small sterile testicular cord had developed. In another 108-day old freemartin, whose gonads contained structures which were interpreted as « forming cords » (Jost *et al.*, 1973*a*), a very small number of meiotic germ cells were found besides a few oogonia. In these two cases inconspicuous and very poorly developed testicular-like structures were seen in gonads also showing one or more meiotic cells. However, it should be recalled that testicular-like structures may develop in the freemartin gonad long after the onset of meiosis.

TABLE 2

Meiosis in freemartins : 3 special cases

Age	Condition of the gonads	Meiosis
93	Right gonad * = no seminif. cords	11 p. 100 LZ
	Left gonad * = many seminif. cords	No meiosis
108	Right gonad = probably forming cords	1.5 p. 100 LZ
	Left gonad = probably forming cords	1.3 p. 100 LZ
146	Right gonad = one single small seminif. cords	one single P. cell (not in the cord)
	Left gonad * = numerous cords	no meiosis

* Animal included in table 1.

C) *Comments.*

Several questions are raised by these observations : why is meiosis incompatible with the presence of seminiferous cords ; why are meiotic changes completely absent from all gonads over 133 days old ? It has been suggested (Jost, 1970, 1972 ; Jost *et al.*, 1974) that the fetal Sertoli cells could prevent meiosis ; one could speculate that some testicular factor inhibits meiosis or provokes the degeneration of the meiotic cells ; one may also question whether the factor involved is the testicular Müllerian inhibiting factor. The available evidence is not conclusive : out of six freemartins aged 93 to 115 days, three had heavily inhibited Müllerian ducts (tubes and uterine horns absent), but only one was deprived of meiosis ; three others had less inhibited Müllerian ducts (uterine horns largely persisting) and in two of them meiosis was absent. If one takes into account the time correlation in the development of freemartins, these observations are not compelling. In freemartins an increase in the number of germ cells is prevented as soon as day 54, whereas suppression of the uterine horns occurs later.

The data summarized in table 1, also suggest that freemartin gonads which develop seminiferous cords after day 90 or 100 may well have contained meiotic germ cells some weeks before.

It is very important to recall that the germ cells present in the freemartin gonads disappear prenatally. Germ cells were found until the age of 188 days in gonads which had not formed seminiferous cords. This is also the stage when the last oogonia disappear in the control ovaries. In the gonads containing seminiferous cords the germ cells located in these cords survived one more month (table 1). The survival of the germ cells seems to be prolonged if they are enclosed in the seminiferous cords. In any case these germ cells disappear ; since no characteristic leptotene or zygotene aspects are seen, they seem to disappear without entering the meiotic process. The factors responsible for their death remain to be determined.

Part two : Preliminary experiments on fetal rat ovaries *in vitro*

In order to study experimentally humoral influences on germ cells and on meiosis, a simple technique was used (with the help of Mrs O. Valentino). Fetal rat ovaries were cultivated *in vitro* in a synthetic medium (medium 1066, Difco) with no fetal serum added (Rivelis *et al.*, 1976). In ovaries transplanted on day 14, leptotene and zygotene phases became clearly recognizable on day 18 and meiosis occurred thereafter.

Cultures with or without experimental additions (table 3) were studied after 3 days (corresponding to an intra-uterine age of 17 days) or after 4 to 6 days (corresponding to 18-20 days *in utero*), in order to verify whether meiosis could be either anticipated or prevented.

The substances assayed so far (table 3) include oestradiol, an anti-estrogen (CI-628 Parke-Davis), progesterone, and methyladenine (6 methylaminopurine), a substance acting on the final stages of meiosis in starfish.

We also used rete fluid from an adult ram (kindly provided by Dr. M. Courot), rete fluid plus testosterone, and two preparations of « inhibine » extracted from ram

rete fluid by Dr. P. Franchimont who kindly gave the samples. Two samples of Müllerian-inhibiting hormone obtained from incubated calf or rat testes interfered with the development of the cultured ovaries (one was a gift from Dr. N. Josso and the other was obtained in the laboratory by one of us, B.V.). The latter assays will be repeated

TABLE 3

Effects on meiosis in fetal rat ovaries transplanted in vitro *on day* 14.5 (medium 1066)

Controls * : day 17.5 — first leptotenes day 18.5 — zygotenes and pachytenes	
Estradiol : 10^{-9} M and 10^{-6} M	No anticipation (on day 17) No prevention (on days 18-20)
CI-628 (Parke-Davis) : 10^{-5} M and 10^{-4} M	
Progesterone : 2.10^{-6} M	
Methyladenine : 10^{-5} M and 10^{-4} M **	
Ram rete fluid 20 p. 100	
Ram rete fluid 20 p. 100 + testosterone 10^{-5} M	
« Inhibine » (Franchimont) : 368-906 mg/ml ***	

* C. Rivelis *et al.* (1976).

** At 10^{-3} M disappearance of the germ cells.

*** At 1 780 mg/ml disappearance of the germ cells.

in the near future with other preparations. The other compounds assayed did not anticipate meiosis on day 17 and did not prevent the occurrence of meiotic stages on days 20 or later ; no quantitative study has been made so far. The nature of the substances which influence meiosis in the fetal germ cells remains to be discovered.

The study of the substances released by fetal Sertoli cells or any other humoral agent active in freemartins which act on germ cells, is an exciting field of research.

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Résumé. Dans les gonades de fœtus mâles et femelles de bovins et de fœtus free-martins, les cellules germinales peuvent présenter des aspects nucléaires ressemblant à ceux décrits dans d'autres espèces comme étant le stade préleptotène de condensation chromosomique. La plupart de ces aspects se produisent entre 60 et 80 jours après insémination. Le devenir des cellules présentant cet aspect n'est pas connu et il n'est pas certain que cet état nucléaire fasse réellement partie de la méïose.

Si on tient compte des figures reconnues d'une manière plus conventionnelle (leptotène ou zygotène), la méïose a été observée chez trois fœtus free-martin âgés de 77 à 88 jours, mais le pourcentage de cellules méïotiques était faible.

Chez les fœtus plus âgés que 90 jours, aucune cellule méïotique n'était présente dans les gonades dans lesquelles des cordons séminifères étaient développés. Dans les gonades qui ne contenaient pas de cordons séminifères, les figures méïotiques n'ont été vues que chez trois animaux ayant moins de 133 jours ; la méïose n'a pas été observée chez les animaux plus âgés.

La présence de cordons séminifères dans la gonade est incompatible avec la présence de cellules germinales méïotiques. Dans tous les cas, les cellules germinales disparaissent

de la gonade free-martin après le 186e jour, sauf pour quelques cas où elles persistent jusqu'au 222e jour car elles étaient localisées dans les cordons séminifères.

Pour essayer de voir si la méïose pouvait être influencée par différents agents, des ovaires de fœtus de rat ont été cultivés *in vitro* avec ou sans œstradiol, un anti-œstrogène, de la progestérone, de la méthyladénine, du liquide de rete testis de bélier ou un extrait « inhibine » de ce liquide de rete testis. Dans ces expériences préliminaires et non quantitatives, aucune modification de la méïose n'a été remarquée.

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