

SEX CHROMATIN FORMATION IN THE BLASTOCYST OF THE ROE DEER (*CAPREOLUS CAPREOLUS*) DURING DELAYED IMPLANTATION

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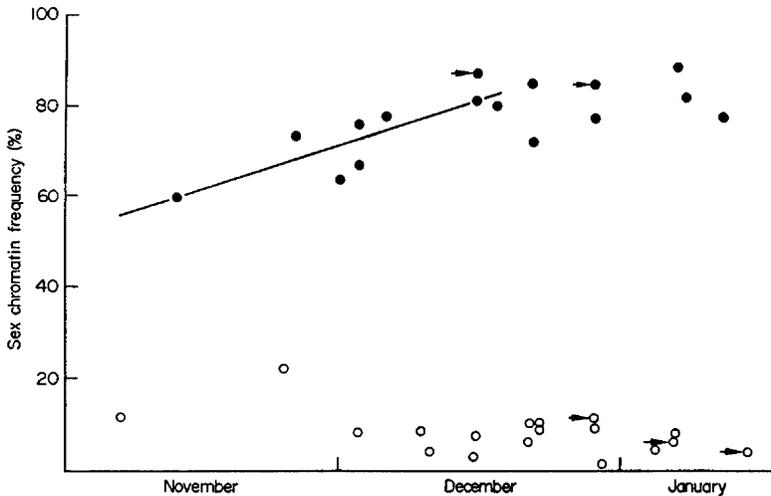
According to the Lyon hypothesis (Lyon, 1961, 1972), random genetic inactivation of one of the two X chromosomes occurs in the somatic cells of female mammals at an early stage of embryonic development. Certain properties of this inactivated chromosome, such as its late replication during the S-phase of mitosis (Taylor, 1960; Grumbach & Morishima, 1962; Mukherjee & Sinha, 1963), heteropyknosis during prophase and the formation of a sex chromatin body during interphase (Ohno, Kaplan & Kinosita, 1959; Ohno & Hauschka, 1960) have been used as criteria for establishing when the process of inactivation begins. These features first appear in blastocysts at about the time of implantation in the cat (Austin & Amoroso, 1957), dog (Austin, 1966), rat (Zybina, 1960), hamster (Hill & Yunis, 1967), vole (*Microtus agrestis*) (Lee & Yunis, 1971), rhesus monkey (*Macaca mulatta*) (Park, 1957) and man (Glenister, 1956; Park, 1957). Gardner & Lyon (1971) used X-linked markers to show that X-inactivation had already occurred in some cells obtained from 3½-day mouse blastocysts before implantation and transferred to host embryos of the same age. In the rabbit (Melander, 1962; Issa, Blank & Atherton, 1969; Plotnick, Klinger & Kosseff, 1971) and pig (Axelson, 1968), sex chromatin formation occurs even earlier, during the morula–blastocyst transition phase. Furthermore, Issa *et al.* (1969) discovered that a late replicating X chromosome can be identified in rabbit morulae about 24 hr before the first appearance of sex chromatin; hence, the formation of sex chromatin may be a secondary phenomenon, occurring some hours after X inactivation.

An analysis of the factors controlling the onset of sex chromatin formation in the rabbit (Klinger, Kosseff & Plotnick, 1971) suggested that absolute time from fertilization was the most important single factor, although a lowering of cell metabolism may also be involved (Klinger, Davis, Goldhuber & Ditta, 1968; Issa *et al.*, 1969). It was, therefore, of interest to study the formation of sex chromatin during the development of a species such as the roe deer in which the blastocyst is maintained unattached and metabolically inactive in the uterine lumen for about 5 months (Short & Hay, 1966; Aitken, Burton, Hawkins, Kerr-Wilson, Short & Steven, 1973).

This study was based upon the analysis of thirty blastocysts and five elonga-

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ting embryos flushed from the uteri of thirty roe deer shot during the annual Forestry Commission cull at Thetford Chase, Norfolk. Whenever possible, the diameter of blastocysts was measured at the time of recovery, before they had started to collapse. The embryos were quickly removed from the flushing medium (0.85% phosphate-buffered saline), fixed in methanol and prepared as flat mounts (Moog & Lutwak-Mann, 1958) for staining by the Feulgen or aceto-orcein technique. The frequency of sex chromatin-positive cells was assessed by scoring at least 100 cells in each embryo; all observations were confined to the trophoblast since little nuclear detail could be distinguished within the inner cell mass of flat mount preparations.



TEXT-FIG. 1. Frequency of sex chromatin-positive cells in roe deer blastocysts recovered during delayed implantation. ○, Presumptive males; ●, presumptive females. Arrows indicate rapidly elongating embryos. A regression line describing the relationship between sex chromatin frequency and recovery date (12th November = 1) for the first nine blastocysts is given by the formula $y = 58.3 + 0.67x$, the correlation being highly significant ($r = 0.85$, $P < 0.001$).

The results of this investigation revealed that the embryos could be divided into two distinct groups on the basis of nuclear morphology (Text-fig. 1). In one group, the presumptive females, a large chromatin condensation resembling the sex chromatin body was observed in a majority of the trophectoderm, and some trophendoderm, cell nuclei. The sex chromatin was normally spherical, U-, or V-shaped and was located adjacent to the nuclear membrane, free in the nucleoplasm or in association with the nucleoli. The mean percentage frequency of sex chromatin-positive cells for all presumptive female embryos was $77.2 \pm 8.3\%$ (mean \pm S.D.). If only those nuclei possessing a sex chromatin body adjacent to the nuclear membrane were scored as positive, the average frequency would have been about 51% (examination of 3000 nuclei from five blastocysts revealed that in 65% of the positively scored cells the sex chromatin was associated with the nuclear membrane). The results presented in Text-fig. 1 also suggested that the sex chromatin frequency was still increasing amongst blastocysts recovered before the 2nd week in December; after this date, the values appeared to level

off. Hence, the frequency observed before 10th December ($69.1 \pm 7.1\%$; mean \pm S.D.) was significantly lower ($P < 0.001$) than the mean observed after that date ($81.6 \pm 5.7\%$; mean \pm S.D.). A positive correlation ($P < 0.01$; $r = 0.87$) was also observed between the frequency of sex chromatin and the diameter of six presumptive female blastocysts (Table 1).

In the second group of embryos, the presumptive males, large condensations of chromatin resembling sex chromatin were only observed in $7.6 \pm 4.5\%$ (mean \pm S.D.) of the cells. In most of the nuclei, fine particles of chromatin could be seen and although they were often associated with the nuclear membrane or nucleoli, these condensations were much smaller than the sex chromatin of female embryos.

On the basis of nuclear morphology, the thirty-five embryos examined could be divided presumptively into eighteen males and seventeen females, which is not significantly different from the expected 1 : 1 sex ratio.

Table 1. The relationship between the diameter and sex chromatin frequency of roe deer blastocysts

<i>Date of blastocyst recovery</i>	<i>Greater diameter of blastocyst (mm)</i>	<i>Sex chromatin frequency (%)</i>
12th November	1.0	59.5
25th November	1.7	72.5
2nd December	2.2	66.7
17th December	2.4	79.6
21st December	4.8	84.5
6th January	9.9	88.6

Since the roe deer has an 'original' type of X chromosome (Gustavsson & Sundt, 1968), it is unlikely that the nuclear condensations observed were of constitutive chromatin such as appears during the cleavage stages of mammals with composite X chromosomes (Hill & Yunis, 1967; Lee & Yunis, 1971) and hence the presence of sex chromatin presumably represents X chromosome inactivation. The results indicate that the frequency of sex chromatin-positive cells increases slowly during delayed implantation, reaching a maximum of about 82% in the middle of December, by which time the blastocyst is about 5 months old; in contrast, 85 to 90% of rabbit blastocyst cells are sex chromatin-positive by 140 hr *post coitum* (Klinger *et al.*, 1971). The gradual formation of sex chromatin in the roe deer blastocyst during diapause may be due to the low rate of cell division at this time; only one dividing cell/1000 can be detected in the roe deer blastocyst at the beginning of December (R. J. Aitken, unpublished observations) compared with a figure of 11/100 in the preimplantation rabbit blastocyst (Moog & Lutwak-Mann, 1958). The formation of sex chromatin during diapause also infers that this process can occur in the absence of the endometrial secretions, which are not released into the uterine lumen until the resumption of rapid embryonic growth in January (Aitken *et al.*, 1973; Aitken, 1974). The irrelevance of specific maternal factors in the initiation of X chromosome inactivation is also suggested by the fact that sex chromatin formation

occurs during the culture *in vitro* of rabbit (Klinger *et al.*, 1971) and mouse (De Mars, 1967) embryos.

The roe deer seems to be similar to the rabbit and pig, therefore, in that sex chromatin formation occurs well before implantation. These preliminary results suggest a need for further studies to establish the time at which sex chromatin first appears in this species and its relation to the onset of embryonic diapause. The possibility also arises of using the roe deer blastocyst as a model for investigating the relationship between sex chromatin formation and changes in cell number, cell density and cell division, all of which show a gradual increase during the 5 months of delayed implantation.

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