Possible influence of menstrual cycle on lymphocyte X chromosome mosaicism

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Abstract

Purpose Estrogens are known to selectively influence cell proliferation. Physiological variations of blood hormone concentration might play a role in regulating the level of X chromosome aneuploidy. In this study we observed the percentages of X aneuploid cells in standard lymphocyte cultures from blood samples obtained in relation to the menstrual cycle, noting whether collection occurred during either the follicular or the luteal phase.

Methods A study consisting of 28 women with X mosaicism and recurrent pregnancy loss, and 28 age-matched healthy controls. Cytogenetic studies were carried out on peripheral blood samples according to standard procedures.

Results A significant difference in the percentage of X aneuploidy was found in blood samples obtained during different

phases of the menstrual cycle. In the case group, the mean value of aneuploid cells in the follicular and luteal phase samples was 10.0 and 6.3 % respectively and in the control group, it was 2.8 and 1.0 % ($P<0.0001$). The difference in the case group varied between 0 and 8 % (3.6 ± 2.1 %) and in the control group between 0 and 4 % (1.7 ± 1.1 %). The specificity for detecting true X mosaicism was 0.875. We estimate that the initial diagnosis of X mosaicism could be correct in 68 % of patients with recurrent pregnancy loss.

Conclusions This observational study establishes that the time of blood sampling in relation to the menstrual cycle can influence lymphocyte X chromosome mosaicism. The results, further proven by additional controlled studies, would have practical implications for genetic counselling and fertility treatment.

Capsule In women with X mosaicism and recurrent pregnancy loss a significant difference in the percentage of X aneuploidy was found between follicular- and luteal-phase blood samples.

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Introduction

In standard lymphocyte cultures, a low percentage of metaphases with an abnormal number of sex chromosomes can routinely be detected. The result could be interpreted either as low level X mosaicism, which could be age-related, or as a technical artefact [1–3].

This abnormal karyotyping result that identifies the X chromosome mosaic state is not well defined. Moreover, there is no consensus on the definition of what constitutes low level X mosaicism. Some authors consider it to be the presence of ≤ 10 % of aneuploid cells [4–6], others the presence of < 6 % of aneuploid cells [7]. It can be found in women with premature ovarian failure, infertility or a history of recurrent pregnancy loss [4–6, 8–14]. In the general population X chromosome loss correlated with age has been described [1, 3, 15]. The mean

value of X monosomic cells increases from 1.5 % in <5-year-old girls to 5.1 % in women aged >90 years [1].

Cell culture conditions may have an influence on cell proliferative activity and could lead to cell transformation. Estrogens are known to selectively influence cell proliferation and could affect X chromosome aneuploidy [16,17]. The exposure of Syrian hamster embryo cells to 17 β -estradiol over the concentration range of 0.3–10 μ g/ml for 24–48 h increased the percentage of aneuploidy metaphases in a dose-related manner. No structural abnormalities were found in the karyotypes, but chromosome losses or gains in the range of 28 % were observed in all cultures treated with 0.3–6 μ g/ml 17 β -estradiol [16].

Horsman et al. [18] also demonstrated that the frequency of X aneuploid metaphases can vary if an estrogen component is present in culture media. Even more, they suggested that in vivo, physiological variations of hormone concentration might play a role in regulating the level of X chromosome aneuploidy. Therefore, the estrogen/progesterone hormone balance of the patient at the time of blood collection may potentially influence lymphocyte proliferation and mitotic behaviour [18]. In the available literature, we did not find any information about the recommended timing (in relation to the menstrual cycle) of taking blood samples for subsequent karyotyping.

The aim of the present study was to observe the percentages of X aneuploid cells in standard lymphocyte cultures from blood samples obtained in relation to the menstrual cycle, noting whether collection occurred during either the follicular or the luteal phase.

Materials and methods

Participants

Fifty-six women were prospectively enrolled in the study during January 2008 and December 2012 at the Department of Obstetrics and Gynaecology (University Medical Center Ljubljana, Slovenia). All women gave their informed consent. The study design was approved by the National Medical Ethics Committee of the Republic of Slovenia (No.39/02/05).

The case population consisted of 28 women with X chromosome mosaicism. X chromosome mosaicism was diagnosed when an abnormal karyotyping result with ≥ 6 % aneuploid cells from at least two cultures of lymphocytes using routine G-banding chromosome analysis was found. These 28 women represent all the cases diagnosed as having X mosaicism in a random sample of 332 women with a history of recurrent pregnancy loss in the five-year-period of the study. A history of recurrent pregnancy loss was defined as two or more consecutive pregnancy losses before 22 weeks of gestation without any previous successful deliveries. The average number of miscarriages was 3.4, ranging from 2 to 5. Only in patients with three or more pregnancy losses, tissue samples

were routinely obtained during the evacuation of the products of conception (missed or incomplete abortion) and transported to the cytogenetic laboratory for karyotyping. Chromosome abnormalities were found in 8 (50 %) out of 16 chorionic villi samples (trisomy 15, 21 or 22, monosomy X, mosaic 47,XX,+16/46,XX or 47,XXY/46,XX).

All participants had had a regular menstrual cycle since the last pregnancy loss (29.0 ± 1.6 days; range 27–33 days). Patients with uterine malformations, chronic vascular, renal or autoimmune diseases were excluded.

Twenty-eight age-matched women participated voluntarily as a control group. They were randomly selected from the outpatient clinic records of the Department of Obstetrics and Gynaecology (University Medical Center Ljubljana, Slovenia). They had regular menstrual cycles for the duration of at least 1 year (28.8 ± 1.1 days; range 27–31 days), at least one healthy child, and no history of infant abnormality, uterine malformations, and chronic vascular, renal or autoimmune diseases. All women had a normal initial karyotype (≤ 5 % of X aneuploidy).

In all 56 participants of Caucasian ethnic origin, the results of thyroid evaluation (TSH, T3, T4, and anti-thyroid antibodies), FSH, adrenal hormones and a comprehensive biochemistry panel (including calcium, phosphorus, electrolytes, cholesterol, and fasting glucose) were normal. They had normal BMI (from 18.5 to 25 kg/m²), and the mean age was 30.5 years (SD 6; range between 20 and 39 years) in both groups. At least 1 month before entering the study their complete blood cell count, haematocrit and erythrocyte sedimentation rate were normal according to the ranges provided by the laboratory. There were no clinical signs of inflammatory disease during blood sampling for karyotyping.

The ovulatory pattern of follicular phase was observed by transvaginal ultrasonographic scans (GEHealthcare, Voluson VE8 Expert, endocavity transducer RIC6-12-D 5–13 MHz). The size of dominant follicles was determined by measuring one diameter in the case of a round follicle and calculating the mean of two diameters if the follicle was oval [19,20]. Two scans were performed; the first scan at the end of menstrual bleeding (day 5–7 of menstrual cycle) and the second one 4 to 5 days later (day 9–12 of the cycle). The images were photographed and stored. If the dominant follicle was not clearly detected, the ultrasonographic observation and blood sampling were postponed until the next cycle.

Cytogenetic analysis

Cytogenetic studies were carried out on peripheral blood samples. In relation to the menstrual cycle, samples were taken at two times, during the follicular phase (day 9–12 of the cycle) and during the luteal phase (1–4 days before menstrual bleeding). Aliquots of white blood cell suspension were placed into six flasks and cultured in Chromosome Medium

with Phytohaemagglutinin (Life Technologies, GIBCO, USA) for approximately 72 h. The cultures were harvested and slides prepared by standard cytogenetic techniques [21].

For each chromosome analysis, the first 100 successive G-banded cells were counted and analysed to exclude the presence of 5 % mosaicism with a 0.99 level of confidence [12,22]. At the same time, structural and numeric abnormalities of all chromosomes were analysed. The X chromosome mosaic state was diagnosed when the number of aneuploid cells represented 6 % or more of the total number of metaphases analysed, and when the aneuploid cells were found in two or more cultures [23].

When X chromosome mosaicism was diagnosed, the level of X aneuploidy was defined as well. The presence of more than 10 % of aneuploid cells was considered true level X mosaicism, whereas low level X mosaicism was defined as 6–10 % of aneuploid cells [6]. X chromosome mosaic state was excluded when less than 5 % of aneuploid cells were present in blood samples from both the follicular and luteal phases.

Statistical analyses

Means, standard deviations and ranges were used to describe the distribution of the continuous variables; counts and proportions were reported for the binary covariates. T tests were used for the comparison of continuous variables. Data was graphically checked for any important departures from normality.

All estimates of population values were accompanied with 95 % confidence intervals. The association between continuous variables was checked using linear regression. The specificity of X mosaicism diagnosis, without taking into account the menstrual cycle phase, was estimated under the assumption that the probability of X mosaicism being measured in each of the phases is 0.5. The 95 % bootstrap confidence intervals accompany our estimate. The analysis was performed using R software for Windows, version 2.15 [24].

Results

During the follicular phase, blood samples were taken on day 11.3 ± 0.4 of the cycle (range 9 to 12 days; on day 9 only one woman from control group). During the luteal phase, samples were taken 2.3 ± 0.5 days before the beginning of menstruation (range 1 to 4 days before beginning of menstruation; 4 days before menstruation in 4 women - 2 from each group).

The percentage of X aneuploidy was significantly different in lymphocyte cultures from blood samples obtained in relation to the menstrual cycle between women from case and control groups (Table 1). In addition, in both groups, the

percentages of X aneuploidy were higher when blood samples were taken during the follicular phase, compared with the luteal phase. In the case group, the mean value of aneuploid cells in the follicular and luteal phase samples was 10.0 and 6.3 % respectively and in the control group, it was 2.8 % and 1.0 % ($P < 0.0001$).

True or low level X mosaicism was detected only in the case group (Fig. 1); six or more percent of aneuploid cells were present in all 28 samples from the follicular and in 14 samples (14 out of 28; 50 %) from the luteal phase. More than 10 % of aneuploid cells (true level mosaicism) were present in 8 out of 28 samples and 6–10 % of aneuploid cells (low level mosaicism) were present in 20 out of 28 samples from the follicular phase. In only one woman out of 8 with true mosaicism in the follicular phase, the number of aneuploid cells was reduced to low level mosaicism in the luteal phase (1 out of 8; 12.5 %). In 7 out of 8, the percentage of aneuploid cells has remained more than 10 in both phases. X aneuploid cells were found in 2.82 cultures of each sample (SD 0.57, range 2 to 6); there were no other chromosomal abnormalities observed.

In the control group the aneuploid cells were present in 0–5 % of samples from the follicular and in 0–3 % of samples from the luteal phase (Fig. 1).

The differences in percentage of X aneuploidy between follicular and luteal phase samples are presented in Table 1 and Fig. 1. In the case group, the difference in the mean percentage of aneuploid cells varied between 0 and 8 % between the follicular and luteal phases and it is highly statistically significant ($P < 0.0001$). The 95 % confidence interval (CI) for the mean difference equals [2.8,4.5]. The difference between the follicular and luteal phase is highly statistically significant also in the control group (0 and 4 %) ($P < 0.0001$; 95 % CI [1.3,2.1]). The difference in phases exists when the results of all women are compared.

There were 5 women out of 56 (0.089, 95 % CI [0.03,0.196]) in which no difference was found between the follicular phase and luteal phase karyotypings. Four of these women were in the control group (three had a value of zero in both phases) and one was in the case group (0.018, 95 % CI [0.001,0.183]).

There were no women in either the case or control groups, whose percentage of X aneuploidy in the luteal phase was measured to be larger than the percentage in the follicular phase (Fig. 1). Since our sample contains only 56 women, we can conclude (with 95 % confidence) that the actual proportion of such women is less than or equal to 0.064.

The expected difference between the blood samples in relation to the menstrual cycle depends neither on the value in the luteal phase (estimated coefficient 0.03, $P > 0.5$, 95 % CI [−0.16,0.2]) nor on the age (estimated coefficient −0.01, $P > 0.5$; 95 % CI [−0.86,0.83]).

Table 1 The mean value of aneuploidy cells in lymphocyte cultures from blood samples obtained in relation to the menstrual cycle

	Mean value of aneuploid cells (%) [SD; range]		
	Case group (n=28)	Control group (n=28)	All women (n=56)
Follicular phase	10 [4.4; 6–20]	2.8 [1.5; 0–5]	6.4 [4.9; 0–20]
Luteal phase	6.3 [4.8; 0–15]	1 [1.1; 0–3]	3.7 [4.4; 0–15]
Difference	3.6 [2.1; 0–8]	1.7 [1.1; 0–4]	2.7 [1.9; 0–8]
P	<0.0001	<0.0001	<0.0001

The level of X aneuploidy was found as true mosaicism in one phase, but as low in the other for one woman, which represents 12.5 % of all cases with true level X mosaicism (8 women). Assuming that the proportion of true X mosaicisms not detected in our sample population (women which were not included as cases in our study but would have the test result above 10 % in the follicular stage) is negligible, the specificity of our test was 0.875 (95 % CI [0.473,0.997]).

Discussion

We observed a significant difference between the percentages of X aneuploid cells in lymphocyte cultures from blood samples obtained during the follicular phase and during the luteal phase. As far as we know, this is the first data on possible physiological variations of X aneuploidy in the female population in relation to the time of sampling.

Although fluorescence in situ hybridization (FISH) may be the most appropriate method for detecting low level X chromosome mosaicism [9,14] we evaluated the presence of aneuploid metaphases by routine G-banding chromosome analysis, in order for the results to be comparable with some previous studies [5,6,12]. However, according to the International System for Human Cytogenetic Nomenclature (ISCN), numerical and structural abnormalities still have to be excluded at a banding level appropriate to the referral's guidelines [1,25,26].

All our participants had had a regular menstrual cycle (case group 29.0 ± 1.6 days, range 27–33 days; control group 28.8 ± 1.1 days, range 27–31 days), which reduces the impact of various cycle lengths.

Due to limited financial resources estrogen levels were not measured at the same time of blood collection for karyotyping. The clinical part of the research protocol was designed on the assumption of a good correlation between the diameter of the dominant follicle and serum concentration of estradiol [19,20,27,28]. During the follicular phase, a blood sample was taken only when the dominant follicle was visible. Otherwise, the ultrasonographic observation was postponed to the next cycle. We have assumed that the level of estradiol in the late luteal phase was lower than between days 9–12 of the menstrual cycle, when dominant follicles were clearly visible by transvaginal scans.

In the follicular phase, the growing follicles, and later the dominant follicle, secrete an increasing amount of estradiol. Estrogens interact with microtubules and may inhibit the polymerization of tubulin to form microtubules. Differences in chromosome/spindle interactions of individual chromosomes may mediate mitotic arrest and aneuploidy can be induced through the process of non-disjunction, or through chromosome loss [17,29]. In Chinese Hamster cell cultures, different estrogens (17 β -estradiol, ethinyl estradiol, dienestrol, estriol) caused a significant increase in aneuploidy within a narrow range of hormonal concentrations [17]. A similar finding was reported in different cell cultures exposed to 17 β -estradiol [16,29,30].

Fig. 1 The percentages of cells with abnormal number of X chromosomes in lymphocyte cultures from blood samples obtained in relation to the menstrual cycle; **a** case group of women with X chromosome

mosaicism, **b** control group. Pair of open circles connected with a line indicates the values of aneuploid cells in two different samples taken from each individual woman

Because estradiol levels vary between the two phases of the menstrual cycle, we assumed, according to Horsman's findings [18], that the patient's estrogen status at the time of blood collection might have a predominant influence on the lymphocyte proliferation and mitotic behaviour without the potential impact of "history of cumulative doses of estrogen exposure".

On the other hand, the estradiol/progesterone balance during the menstrual cycle can mediate immunity and have an impact on the proportions of B and T lymphocytes [31,32]. It might also have an impact on their mitotic behaviour. Further research is required to assess this assumption.

Only women diagnosed with X chromosome mosaicism were included in the study. There was no data in the literature available about whether the phase of the menstrual cycle at the time of routine blood sampling for karyotyping has any influence on subsequent results; therefore, women were initially, during selection for our case group, karyotyped at random times without respect to their menstrual cycle.

X mosaicism was found in one phase, but not in the other, in 50 % of our cases (14 women). The reason why these women were regarded as cases was that their initial diagnosis was set based on the result of karyotyping in a phase when their percentage was high. If blood samples of these women were taken in the luteal phase instead, they would not be regarded as cases in our study. When designing our study, we limited our measurements to 8 days of the menstrual cycle - we chose the two periods in which the highest difference in the percentage of X mosaicism was expected. Based on the results of our observational study alone, it is thus impossible to predict the values on other days. Subsequently, it is not possible to determine on how many days of the menstrual cycle the values would drop as low as in the luteal phase. The specificity of our diagnosis depends on the proportion of days in the cycle when values are low; the higher this proportion, the lower the specificity of our diagnosis. For example, if we assume that the probability of being karyotyped in either phase (high or low) is equal, we can estimate that only 68 % of the patients were diagnosed correctly (95 % bootstrap CI for the specificity is [0.6,0.76]).

The definition of low level X mosaicism varies in the literature. One of the reasons why there is still no consensus on the "risk threshold" may also lie in the fact that the percentages of aneuploidy measured in different menstrual phases are simply not comparable. We suggest that it is highly probable that any study that used the percentage of aneuploid cells as a predictive factor for reproductive health has underestimated its significance, compared to what the results would show if the blood samples for karyotyping were taken in different phases of the menstrual cycle. The existence of a true threshold (for example in the follicular phase), above which women are at a higher risk of having recurrent pregnancy loss, premature ovarian failure or infertility, would

imply that a study using blood samples obtained in the luteal phase misclassifies a certain proportion of women. In this way, some of the high-risk women would be found to be below the threshold, thus increasing the observed proportion in the low-risk group and decreasing the observed proportion in the high-risk group. This would have the effect of making the two groups seem more similar than they really are.

To conclude, both in women with recurrent pregnancy loss and in normal women, we observed a significant difference in the percentages of X aneuploidy in blood samples obtained in relation to the menstrual cycle. We are aware the interpretation and conclusions are merely assumptions, and for any concrete evidence a more precise observational protocol with hormonal laboratory tests is required. Presumably, the results so obtained would have practical implications for genetic counselling and fertility treatment.

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Conflict of interest The authors declare no conflict of interest.

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