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LETTER TO THE EDITOR

Sperm Biology

Unpaired sex chromosomes in metaphase I human spermatocytes locally modify autosomal bivalents positioning

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Dear Editor,

In interphase nuclei, chromosomes occupy specific localizations called chromosome territories (CTs). Distribution of CTs has been related to chromosome size and gene density and is known to influence gene expression.¹ This pattern of organization appears to be a widespread nuclear feature, both in somatic and germ cells.

In spermatogenic cells, we have previously revealed a somatic-like distribution of bivalents at metaphase I (MI) with a nonrandom bivalent disposition and a preferential association between bivalents according to chromosome size, morphology, and gene density.² Other studies have revealed preferential proximity of chromosomes 15 and 22 to the sex-chromosomes bivalent in MI.³ Moreover, a nonrandom chromosome distribution has also been reported in sperm nuclei.⁴ Although the functionality of CTs in male germ cells is not fully understood, it has been proposed that it has a role in the regulation of gene expression during spermatogenesis and early embryo development.⁵ Accordingly, alterations in CTs in germ cells could have implications for genome regulation leading to male infertility. Our research group has provided the first evidence of alteration in CTs in the presence of Robertsonian translocations (rob) in MI human spermatocytes.⁶ The impaired fertility in rob carriers is mainly due to the production of chromosomally abnormal spermatozoa because of the regular segregation of the chromosomes involved in the reorganization and the occurrence of interchromosomal effects.⁷ However, we have also revealed in rob carriers that, the trivalent configuration of the reorganized chromosomes disturbs the overall chromosome positioning at MI.⁶ Thus, the disruption of MI CTs could be an additional contributory factor to infertility associated with this kind of chromosomal reorganization.⁶

Interestingly, in human spermatogenesis, the XY chromosomes organize in the sex vesicle at prophase I,⁸ and in the postmeiotic sex chromatin (PMSC) body in spermatids.⁹ In terms of gene expression and CTs organization, sex chromosomes configure a silent compartment where chromosomes appear inactivated from the

pachytene stage to round spermatids. Furthermore, it has been reported that sex-chromosome univalency is relatively common in human MI spermatocytes (from infertile males).¹⁰ The pairing features of these chromosomes (restricted to the short pseudoautosomal regions) promote the presence of univalents and explain the high incidence of sex-chromosome aneuploidies in human spermatozoa from infertile patients.¹¹ Nevertheless, the influence of univalency in the territorial organization of human spermatocytes has not been ascertained.

In this study, we have determined the effect of the presence of sex-chromosome univalents on the territorial organization of MI human spermatocytes from infertile patients with a normal somatic karyotype (46,XY).

Chromosome proximity analysis was performed in a total of 1944 chromosome units from 81 MI,24,X,Y (MI bearing the X and Y chromosomes as univalents). MI spermatocytes were obtained from testicular biopsies from twenty-six 46,XY infertile men (range 1–10 MI per individual). Biopsies were obtained for diagnostic purposes to evaluate the incidence of meiotic chromosomal abnormalities in spermatocytes at MI,¹⁰ and surplus material was used for this study. The study was approved by the Institutional Review Board of the collaborating centers (Centre de Reproducció Assistida Fecunmed, Fundació Puigvert, Institut Marquès, Institut Universitari Dexeus and Instituto de Reproducción CEFER). In every instance, all of the patients signed their informed consent about participation in the study and the centers provided all data while preserving patient anonymity.

Testicular biopsies were mechanically disaggregated, and cell suspensions were fixed and dropped onto dry slides.¹⁰ Chromosome preparations were processed following a protocol that combine Leishman staining (PanReac AppliChem, Castellar del Vallès, Spain) and multiplex fluorescence *in situ* hybridization (M-FISH, Spectra Vysion™ Assay Protocol, Vysis Inc., Downers Grove, IL, USA) procedures (Figure 1).¹⁰ Briefly, MI images from Leishman-stained slides were captured and coordinates were noted. Slides were further processed for M-FISH analysis, which enabled unequivocal chromosome identification based on a distinctive color pattern. MI cells were re-located and re-analyzed after the FISH procedure.

Proximity analysis was performed as it has been previously described.² Briefly, in each cell, nearby chromosome units (including bivalents and univalents) were determined for each unit contrasting

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the images obtained after the M-FISH protocol with those previously captured after Leishman staining. We considered nearby units to be those forming the first ring around the scored unit (**Figure 1**). For each MI, 276 proximity relationships are theoretically possible; from 24 chromosome units, there are $C(24, 2) = 1/2 \times 24 \times 23 = 276$ combinations of two without repetitions. Nearby units were scored as “1” and the absence of proximity as “0.” A table of proximity was constructed dividing, for each pair of chromosomes, the number of proximities between the number of metaphases. With the aim to summarize proximities between chromosome units, a multidimensional scaling analysis was performed. Data obtained in this analysis was used in a hierarchical cluster analysis. Results were graphically presented in a tree dendrogram, and the number of clusters was determined using the cubic clustering criterion.¹² Furthermore, the concordance between the clusters obtained in MI,24,X,Y and the model of territoriality reported in MI,23,XY² was evaluated by Cohen's *kappa* coefficient. The concordance was assessed taking into account the percentage of chromosome units which remained in the same cluster and those that changed. Finally, Poisson regression models were used to compare the proximities between representative groups of bivalents according to size, morphology, gene density, and the presence of heterochromatic blocks (see detailed groups in Vergés *et al.*²). The number of metaphases observed per individual was considered as an offset term of the model, and the presence of overdispersion was also taken into consideration. The level of statistical significance was set to 0.05.

The comparison between the clusterization of MI,24,X,Y and MI,23,XY enabled a similarity categorization of “substantial”, with a *kappa* index of 0.647 (95% confidence interval [CI]: 0.419–0.875). The cluster formed by the largest chromosomes (bivalents 1 to 12) was conserved between MI,24,X,Y and MI,23,XY (**Figure 2**). In contrast, the remaining clusters were the result of the regrouping of chromosomal

units that formed separated clusters in MI,23,XY (**Figure 1**). In cluster 2, an association between acrocentric chromosomes (13; 14; 15; 21; 22) and the Y chromosome was observed (**Figure 1**). Cluster 4 only contained the X chromosome, indicating that this chromosome had a heterogeneous proximity relationship with the remaining bivalents (**Figure 1**). The analysis of data according to the representative groups of bivalents in terms of size, morphology, gene density, and the presence of heterochromatic blocks revealed statistically significant nearby locations of acrocentric ($P = 0.019$) and small-size bivalents ($P = 0.030$), in agreement with previous results from MI,23,XY cells.² In contrast, the organization of bivalents based on gene density and large chromosomal size, which were reported in MI,23,XY,² was lost in the presence of sex-chromosome univalents.

Overall, the results suggest that the territorial organization of chromosomes in MI is partially modified by the presence of sex-chromosome univalents. It is important to remark that the X chromosome showed a heterogeneous distribution, while the Y chromosome preserved association with the acrocentric chromosomes. This association could be explained by the homology between the heterochromatic regions of the Y and the acrocentric chromosomes,¹³ which would promote heterologous pairing at prophase I that could be maintained at least until the MI stage. Indirect evidence that supports the association observed between the Y chromosome and acrocentric chromosomes is that 70% of *de novo* reciprocal translocations involving the Y chromosome and any autosome are produced between the heterochromatin Yq and the short arm of an acrocentric chromosome.¹⁴ Moreover, since acrocentric chromosomes form a specific cluster at meiosis I, it could be interpreted

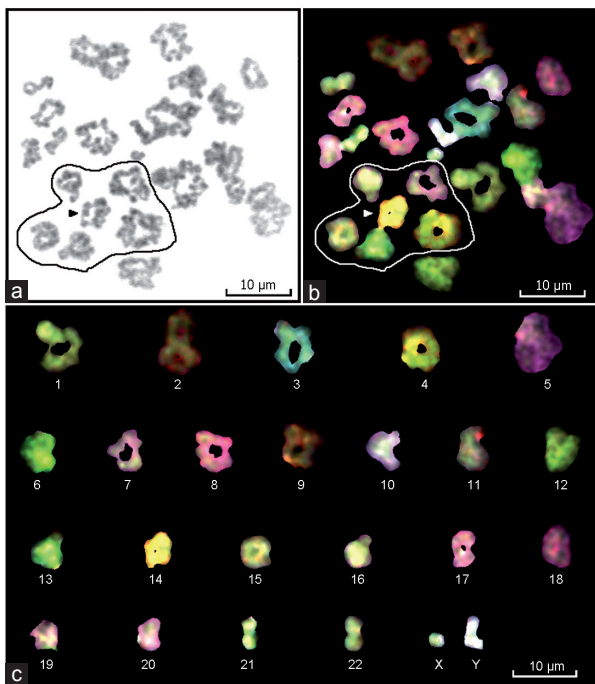


Figure 1: MI in (a) Leishman staining and (b) M-FISH with (c) the corresponding M-FISH karyotype. (a and b) Identification of the bivalents that constitute the first ring around the bivalent 14 (indicated with an arrowhead): 4; 7; 13; 15; 16. MI: metaphase I; M-FISH: multiplex fluorescence *in situ* hybridization.

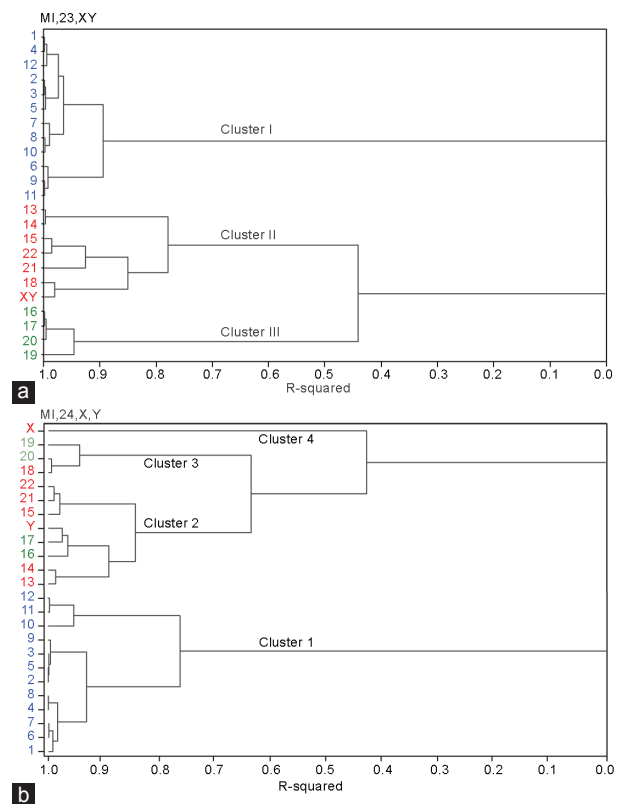


Figure 2: Tree diagram of chromosome units proximities. (a) Dendrogram for MI,23,XY.² (b) Dendrogram for MI,24,X,Y. The color code indicates the groups of chromosomes obtained in the cluster analysis of MI,23,XY. Blue: cluster I; Red: cluster II; Green: cluster III; MI: metaphase I.

as a facilitating factor for the formation of *de novo* rob, which are the most common recurrent structural reorganizations in humans.¹⁵

It cannot be ignored that sex chromosomes show distinctive characteristics about size, morphology, and gene density. It has been reported that these characteristics influence bivalent positioning in MI,² and therefore, it is not surprising that the presence of unpaired X and Y chromosomes will promote some alterations in the proximity relationship among other chromosomes.

We should like to note that the most likely destiny of a spermatocyte with univalents would be its elimination due to the activation of the spindle assembly checkpoint. Moreover, X and Y chromosome univalences could also disturb processes of sex chromosome inactivation and compromise meiotic progression throughout alteration of gene expression. Nevertheless, inefficiencies in cell cycle checkpoint could be inferred through the high incidences of sex-chromosome aneuploidies that have been widely described in spermatozoa from infertile males.¹¹ Thus, it could be envisaged that some spermatocytes displaying sex-chromosome univalents and alterations on CTs will progress along spermatogenesis resulting in genetically abnormal spermatozoa that would compromise the fertility of the individual.

AUTHOR CONTRIBUTIONS

JB and ZS conceived, designed and coordinated the study. CM and ZS carried out the experimental procedures. OV performed the statistical analysis. CM, JB, FV, and ZS were involved in data analysis, interpretation, and manuscript writing. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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