School of Biomedical Science

The Effect of Cell Culture on X Chromosome Mosaicism

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Declaration of Authenticity

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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ABSTRACT

Low level mosaicism is an ongoing problem for cytogeneticists interpreting chromosome preparations and is particularly common with the X chromosome. In this study the effects of cell culture on X chromosome aneuploidy were investigated. Seventy eight young women (22-40 years) with established fertility from the local West Australian population were tested for X chromosome aneuploidy using fluorescence in situ hybridisation methods on both cultured and uncultured (direct) lymphocytes. Females with possible fertility problems were specifically excluded from this study since X chromosome aneuploidy is more frequently seen in this cohort.

All participants had <5% X aneuploidy in both cultured and uncultured preparations, with X chromosome loss occurring (2.4%) more frequently than gain (0.7%). Cultured preparations had a mean of 2.1% cells with X chromosome aneuploidy (95% CI 1.9–2.3%) compared with a mean rate of 0.9% aneuploidy in uncultured preparations (95% CI 0.7–1.1%). The relative risk for cultured preparations having X aneuploidy compared with uncultured cells was 2.33 ($P < 0.001$) (95% CI 2.1–2.6). It was observed that less aneuploidy occurred in uncultured cells than in cultured cells. While this result is not surprising, it suggests that the incorporation of routine use of uncultured cells may be desirable in applications where fertility and other assessments of mosaicism are being determined. The effect of variation in time that cells spent in culture was also explored. In keeping with many cytogenetic procedures, careful attention to laboratory practice and interpretation of results are required for consistent outcomes.

The results from this study on essentially normal young women provide important information to genetic counselors and cytogeneticists interpreting the low background levels of X aneuploidy seen in routine cytogenetic practice. A <10% cutoff is commonly used for determining significance of low level aneuploidy. This study found that in normal, fertile young women (≤40 years) the rate of X chromosome aneuploidy was lower at <5% than the usually accepted <10% regardless of preparation technique. The results also suggest that it will be diagnostically useful to clarify the effects of chromosome culture on mosaic aneuploidy in other patient groups, including older women and those with infertility.

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ABBREVIATIONS

≈	approximately
AneuP C1	72 hour culture
AneuP C2	96 hour culture
AneuP D	uncultured
ART	assisted reproductive technologies
CEP	centromeric enumeration probe
CI	confidence interval
C1	culture type 1 - 72 hour culture
C2	culture type 2 - 96 hour culture
DNA	deoxyribonucleic acid
FISH	fluorescence *in situ* hybridization
G	gravitational force
hr, hrs	hour, hours
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilisation
IQ	intelligence quotient
Kbp, Mbp	Kilo base pair, Mega base pair
KCL	potassium chloride
M	molar
min	minute
mL, µL	millilitre, microlitre
n	number
NP40	Nonident P-40
PHA	phytohaemagglutinin
RPMI	Roswell Park Memorial Institute medium
R	range
R^2	regression equation
RR	rate ratio
SSC	saline sodium citrate buffer
x	times

CHAPTER 1

LOW LEVEL X CHROMOSOME MOSAICISM IN FEMALES

Routine cytogenetic testing using cultured peripheral blood lymphocytes is associated with low levels of X chromosome aneuploidy that complicates interpretation of results. This phenomenon is usually attributed to "artefactual mosaicism" arising from the in vitro culture methods used, however it is also well established that true constitutional X chromosome mosaicism is more prevalent in women with fertility problems. This study reports the results of X chromosome quantification in both cultured and uncultured cells from women with proven fertility. The data will allow better understanding of the overall significance of low level X mosaicism. Such information will be helpful for counselling women experiencing fertility problems with documented low levels of X chromosome mosaicism.

1.1 Introduction

Cytogenetics involves the study of chromosomes. In humans, there are normally 46 chromosomes or 23 pairs of chromosomes in a diploid cell. The autosomes are chromosome pairs 1-22 and are the same in males and females. The sex chromosomes are normally a pair of X chromosomes in females and X and Y chromosome in males.

Chromosome analysis is a test that is routinely employed by investigators to explore the causes of pathology in a number of different patient groups. It may be used to find causes of developmental delay, especially if associated with mental retardation. It is used to monitor changes in cell lines of patients suffering malignancy, and it is also routinely used to investigate the cause of recurrent miscarriage and infertility.

Routine chromosome analysis involves techniques that utilise light microscopy, where the cells to be analysed are arrested in mitosis at metaphase. These chromosomes are then treated with proteases to create banding patterns then stained to allow visualisation. Each chromosome pair contains a unique sequence of DNA, which allows the chromosome pairs to be identified from one another by their characteristic banding patterns. Cytogeneticists can then determine if there

are any numerical or structural rearrangements of the chromosomes. Chromosome analysis offers a snapshot of the whole cell genome at an optional resolution of approximately 3-5 Mbp (Figure 1.1).

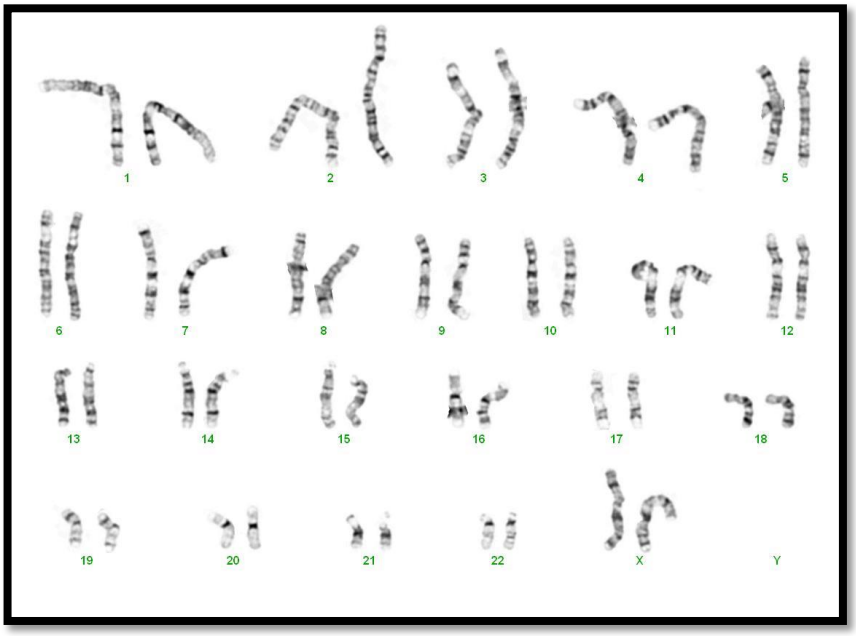


Figure 1.1: Normal female karyotype with G-banding at 400 band resolution.

Increasingly microscopy using fluorescence *in situ* hybridisation (FISH) for specific DNA sequences within the chromosome are being utilised in routine cytogenetic laboratories as an adjunct to chromosome analysis. FISH uses DNA probes that bind to specific sequences on chromosomes in cytogenetic preparations and detects these probes by labeling them with fluorescent dyes. FISH analysis has the advantage that actively dividing cells are not required, as the probes can attach to interphase as well as metaphase cells. FISH also allows examination of a specific area of interest on a chromosome to a much higher resolution than standard cytogenetic analysis. Some disadvantages of FISH are that because the fluorescent probes used are highly specific for the DNA sequence they code for, probe selection must be well targeted to the DNA sequence of interest. FISH also does not give a whole genome overview like the analysis of a standard metaphase cell. Typically FISH allows detection of a ~50 Kbp change within a 1-2 Mbp chromosome band (Figure 1.2).

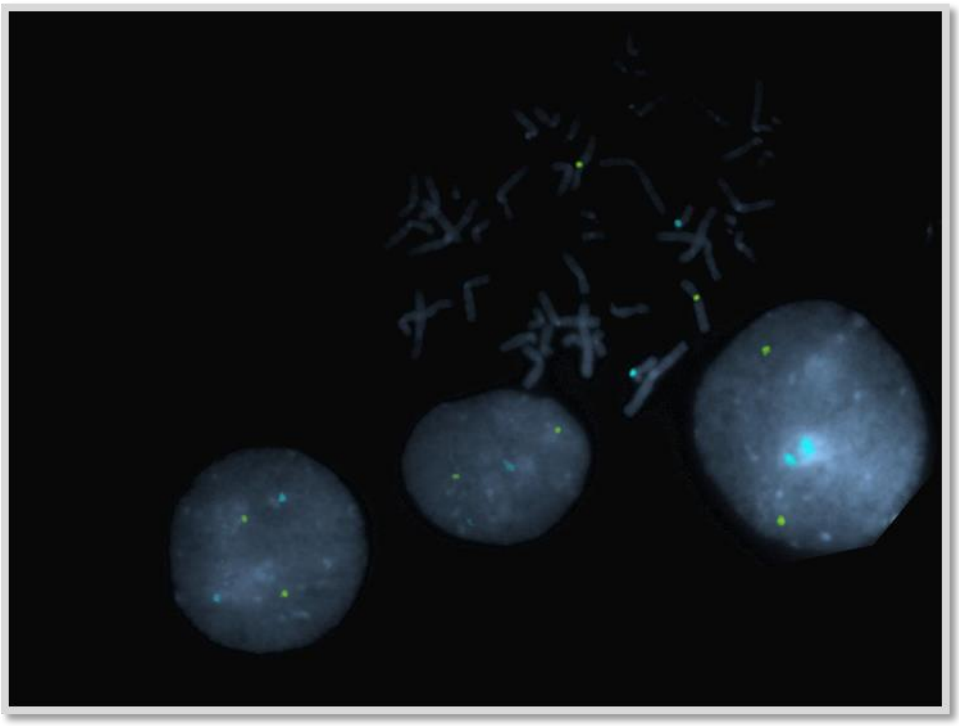


Figure 1.2: An example of FISH staining on interphase nuclei and metaphase chromosomes. As expected two copies of chromosome X (green) and chromosome 18 (blue) can be observed in both stages of cell division.

1.2 Chromosome X Mosaicism

It is the finding of additional, or more commonly missing, X chromosomes in some of the cells analysed from female patients presenting to infertility clinics that has triggered this investigation. Normally women have two X chromosomes in every cell. However variations, of questionable pathological significance, in the number of X chromosomes are occasionally found during cytogenetic analysis. Sometimes a small number of cultured cells are shown to have lost or gained an X chromosome, a phenomenon known as chromosomal mosaicism. For example, if ten percent of cells analysed show loss of the X chromosome, this represents 10% mosaicism (Figure 1.3 and 1.4).

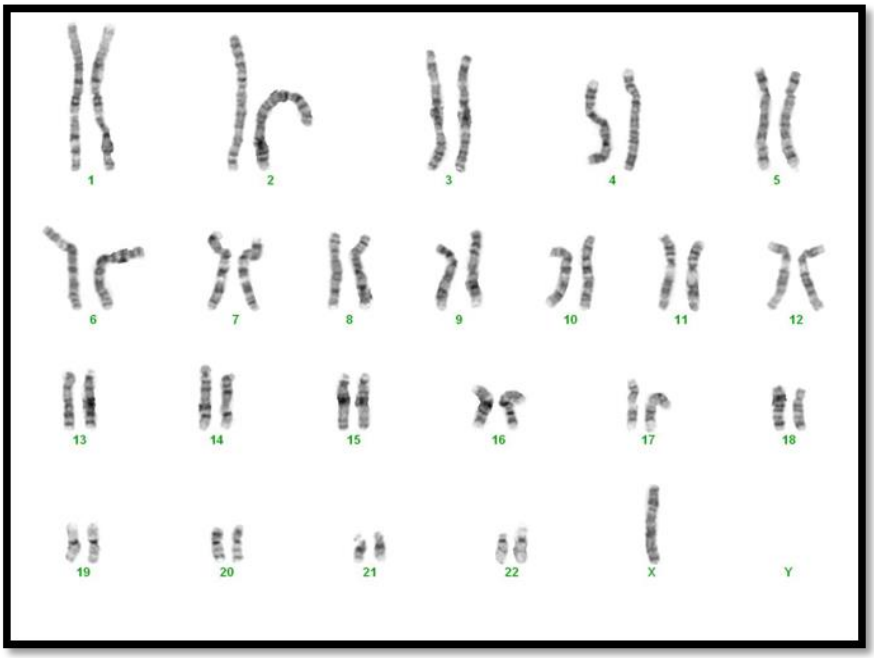


Figure1.3: Monosomy X karyotype – 45,X at 400 band resolution.

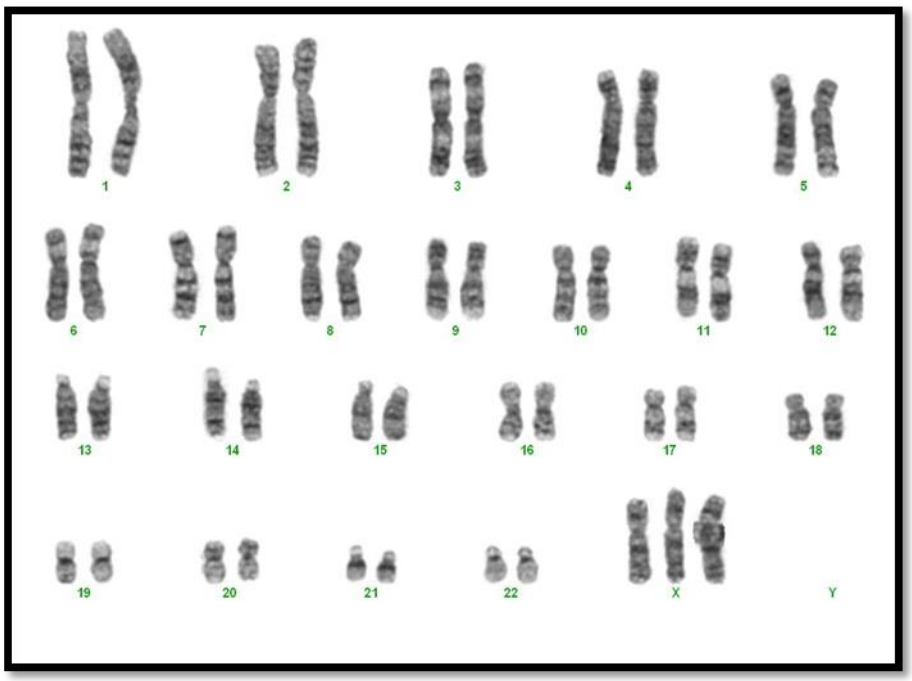


Figure1.4: Triple X karyotype – 47,XXXat 400 band resolution.

Chromosomal mosaicism represents one of the most important problems in cytogenetic diagnosis and genetic counselling (Toncheva, Ilieva and Mavrudieva, 1994). True chromosomal mosaicism is the presence of two or more different cell lines within a single individual (Rooney and Czepulkowski, 1992). Artefactual mosaicism is thought to be likely if the abnormal findings are found only in a few cultured cells, and possibly arises during ex-vivo culture (also known as pseudo-mosaicism). However, distinguishing between true and artefactual mosaicism remains a challenge for cytogeneticists and the findings of low level mosaicism (10% or less) in cultured lymphocytes remain most problematic. There are no clear guidelines established to resolve the effect of cultural processes in producing the mosaicism.

It is recognised that growing cells in culture may alter the proportions of abnormal cells present (Rooney and Czepulkowski, 1992). "Cultural artefact" is the term used to describe findings considered generated merely from culturing processes. In carrying out chromosomal analysis, the cells examined have been stimulated by mitogens to undergo transformation and growth *invitro* and this culturing process may change the proportions of abnormal cells detected (Rooney and Czepulkowski, 1992). The pathological significance of mosaicism present only in the cultured cells of an individual is therefore controversial. There are several factors to consider when evaluating low level X chromosome mosaicism: are the results genuine constitutional mosaicism, cultural artefact or age related? (Wise *et al.,* 2009)

Defining what levels of X chromosome mosaicism are significant and determining the contribution of culture effects on levels of mosaicism, remains one of the most complex and challenging issues (Gersen and Keagle, 1999) in cytogenetic diagnosis and genetic counselling.

1.3 Constitutional X Chromosome Mosaicism and Phenotype

The phenotype of an individual will alter depending on the proportions of cells that have gained or lost X chromosomes. The phenotype of an individual with 45,X was described by Henry Turner in 1938 and subsequently eponymously named after him. Turner syndrome has subsequently been well documented by Sybert &McCauley (2004) amongst many others. The core phenotype consists of short stature, gonadal failure and infertility, reduced IQ compared with siblings, physical defects such as webbed neck and coarctation of the aorta, as well as an increased frequency for some common diseases such as obesity, diabetes

hypertension and heart disease amongst others (Gardner, Sutherland & Schaffer, 2012).

The physical phenotype of a woman with constitutional 47, XXX is generally unremarkable; fertility is uncompromised, although IQ is generally reduced by 10-20 points. Karyotypes with 4 or more X chromosomes are associated with intellectual compromise (Gardner, Sutherland & Schaffer, 2012). The phenotype of an individual with a mosaic karyotype is likely to be variably ameliorated by the percentage of normal female 46,XX cells present.

The association of the Turner syndrome 45,X karyotype and infertility is the cause of consternation during routine cytogenetic analysis when a mosaic cell line is identified. As the majority of women undergoing chromosome testing are presenting for fertility treatments it is difficult to determine if the low levels of aneuploid cells are the cause of any fertility problems or cultural artefact. For women without phenotypic stigmata of Turner syndrome the implication of mosaic X-chromosome aneuploidy remains controversial, particularly in cases with low-level mosaic 45,X/46,XX and/or 47,XXX, as the precise impact of the X-chromosome mosaicism on ovarian function is unknown (Devi *et al*., 1998; Sybert & McCauley, 2004).

Studies of women with mosaic karyotypes (X/XX, X/XXX and X/XX/XXX) have shown that premature ovarian failure is common amongst these women (Devi *et al*., 1998; Blair *et al*., 2001; Sybert *et al*., 2002). Conversely, studies of women with premature ovarian failure have found significant levels of X chromosome mosaicism (Diao *et al*., 2003; Lakhal *et al*., 2010; Baronchelli *et al*., 2011). Tissue specific differences in the level of X aneuploidy have also been demonstrated and may account for differences in phenotype/genotype correlation (Lispenasse *et al*., 1998; Nazarenko, Timoshevskii & Sukhanova, 1999). This may account for the common finding of low level X chromosome aneuploidy in blood cells of women presenting for infertility treatment (Horsman *et al*., 1987; Toncheva, Ilieva and Mavrudieva, 1994; Mau *et al*., 1997; Meschede *et al*., 1998; Scholtes *et al*., 1998; Peschka *et al*., 1999; Gekas *et al*., 2001; Sonntag *et al*., 2001; Morel *et al*., 2002).

1.4 Chromosome X MosaicismIn Cultured And Non-Cultured Cells

Distinguishing between low level X chromosome mosaicism, due to cultural artefact and constitutional X chromosome mosaicism in female patients has always been a problem in cytogenetic investigations. This is especially so when the women being tested are doing so because of fertility problems.
Many factors such as time spent in culture and the type of media used are known to result in changes to the chromosomes subsequently examined. Chromosome loss has been shown to be dependent on culture time, being significantly more frequent in 72 hour than in 48 hour cultures. This increase in chromosome loss resulted essentially from X chromosome loss (Richard F. *et al.,* 1993). Differences between cell division in regard to culture time (48-72hr) have also been demonstrated (Rooney and Czepulkowski, 1992). Cell cycle times of abnormal cells may vary from normal cells and this may change the proportions of X chromosome abnormalities demonstrated (Rooney and Czepulkowski, 1992). The project did not compare the effect of the 72 hr and 96 hr culture times within patients as the effect of time in culture is well documented (Rooney and Czepulkowski, 1992; Richard *et al*., 1993).It has also been shown that different growth media can have a significant cultural effect on X chromosome gain or loss. By creating different culture conditions using various growth media, (Richard F. *et al.,*, 1993; Rooney and Czepulkowski, 1992) variation in results can be expressed. It has been proposed that cells may have different metabolic requirements (Rooney and Czepulkowski, 1992), hence the variation in chromosomal results between media. The effect of media variation will not be further considered in this project.

Another aspect to consider in investigating X chromosome mosaicism is the phenomenon of x inactivation or Lyon Hypothesis. This phenomenon was described by Mary Lyon in 1961, and subsequently eponymously named. It states that one of every two X chromosomes in female mammals is inactivated. X inactivation has subsequently been reviwed by Avner&Heard (2001) The inactive X chromosome is late replicating (Gilbert *et al*., 1962) and this sets the X chromosomes apart from autosome pairs, and thus could be considered a prior null hypothesis, accounting for X specific loss or gain. Abruzzo *et al*., (1985) have shown that the inactive X chromosome is missing in most monosomy X cells which supports this hypothesis.

Numerous studies have demonstrated the increase in incidence of sex chromosome hypodiploidy and the increased incidence of micronuclei formation (Catalan *et al*., 1998; Catalan *et al*., 2000; Bukvic *et al*., 2001; Wodjda and Witt, 2003; Wodjda *et al*., 2006). The X chromosome was highly overrepresented in micronuclei in a study by Catalan *et al*. (1995). Catalan *et al*. (1998) have suggested that the inactive X chromosome shows preferential micronucleation and this is supported by FISH by Guttenbach *et al*., 1994 and Hando *et al*., 1994).

Studies have been performed on lymphocyte and fibroblast cultures, in attempts to distinguish between cultural artefact and (inferred) true constitutional mosaicism. By studying cells with a different embryological origin, constitutional or tissue limited mosaicism can be investigated. Cells of the blood are derived from mesoderm. Buccal mucosa cells and the epidermis of the skin and are derived from ectoderm (the dermis and hypodermis are derived from mesoderm). Both of these tissues have been used to investigate cytogenetic mosaicism.

A significant study on a population of women with 2-10% of X aneuploid cells in cultured lymphocytes, showed no evidence of constitutional chromosome mosaicism based on the analysis of fibroblast cultures (Horsman D. E. *et al.,* 1987). Fibroblast culturing is both time consuming for laboratory processing and is invasive in terms of specimen collection as skin biopsy is required. Buccal mucosal cells are readily available and non invasive to collect. Schad *et al*. (1996) found buccal cells well suited to investigating sex chromosome anomalies using FISH, but claim they are only sufficiently accurate at detecting cell lines of >6%. This finding supports the earlier work by Harris *et al*. (1994) who found high rates of false rates of hypodiploid cells when interrogating buccal cells with FISH probes. They attributed this to karyopyknotic intermediate epithelial cells in the buccal cell samples.

This study compares the presence of possible constitutional mosaicism as inferred by the non cultured interphase FISH cells (after considering inevitable stochastic errors) from cultured FISH cells which may be more likely to be artefactual in origin. As all cultured cells have been stimulated with the same mitogen (PHA), this study has not attempted to disentangle possible effects of mitogen type *per se*, from the effects of culture *per se*.

1.5 Chromosome X Loss and Ageing

The phenomenon of X chromosome aneuploidy in the cultured lymphocytes of aging women is well documented (Fitzgerald, 1975; Ford & Russell, 1985; Nowinski *et al*., 1990; Guttenbach *et al*., 1995; Bukic *et al*., 2001; Russell *et al*., 2007). The rate of X chromosome loss correlates positively with age and increases significantly beyond reproductive age which was defined as greater than 51 years old (Guttenbach *et al*., 1995). Bukic *et al*. (2001) found an approximate 11 fold increase in the number of aneuploid cells in centenarians (98-102 years old) compared to their control group (30 years old). In acknowledgement of this phenomenon women over the age of 40 at the time of blood collection were excluded from this study. Similarly, full karyotyping of each sample tested was not undertaken since chromosome analysis on control age matched women is again well documented (Toncheva*,* Ilieva and Mavrudieva, 1994; Russell *et al*., 2007).

1.6 X Chromosome Aneuploidy in Routine Cytogenetic Testing

At the time of this study, the level of X aneuploidy generally accepted as "normal variation" is currently set at less than 10% mosaicism (Gardner and Sutherland, 2004). However, this guideline was based on data from studying infertile women and there is little evidence of what is usual in fertile women (Horsman *et al*., 1984). Peshka *et al*. (1999) consider low level X chromosome mosaicism as <6% and this figure is supported by the more recent study by Homer *et al.* (2010). They claim that the clinical threshold seems to be 6% for X chromosome mosaicism.

Low level X chromosome mosaicism is known to occur with increased frequency in women presenting for treatment with assisted reproductive technologies (ART) (Horsman *et al*., 1987; Toncheva, Ilieva and Mavrudieva, 1994; Mau *et al*., 1997; Meschede *et al*., 1998; Gekas *et al*., 2001; Sonntag *et al*., 2001; Morel *et al*., 2002; Baronchelli *et al*., 2011). However, the significance of low level mosaicism in this group is poorly understood. It is not clear whether low level X mosaicism contributes to infertility, or is merely a coincidental finding, largely generated by culture processes and not clinically significant with regard to fertility (Voigt *et al*., 2004; Homer *et al*., 2010). Or if it is the result of an ascertainment bias in the population of women who have chromosome analysis performed. Uncertainty remains about what levels of mosaicism should be

considered "significant" and whether results may be influenced by culture process.

Limited studies have been performed on cultured and uncultured lymphocytes to determine if low level X chromosome mosaicismcan be attributed to the lymphocyte culturing process. These studies have looked at 'healthy ' women (but not necessarily those with proven fertility) and also women with Turner syndrome (Nazarenko, Timoshevskii and Ostroverkhova 1997; Nazarenko, Timoshevskii and Sukhanova 1999). However the numbers in these studies are very small and sample type does not reflect routine practice (Nazarenko, Timoshevskii and Ostroverkhova, 1997; Nazarenko, Timoshevskii and Sukhanova, 1999).

1.7 Relevance of this Study

This project is studying women with proven fertility and will determine any variation in the number of X chromosomes that occurs in this population. By comparing lymphocytes that have been through the standard culturing process (*in vitro*) to uncultured lymphocytes on direct blood smears (*in vivo*) as paired samples, we will determine if there is an increase in aneuploidy that can be attributed to the cell culture process and hence clarify the clinical significance of this finding.

In undertaking this project, fluorescence *in situ* hybridisation using centromere-specific DNA probes was employed to identify the number of X chromosomes in each cell. It was determined that the FISH method used would be performed using DNA probes specific for the X chromosome centromere as previously described by Jalal & Law, 1997; Dewald *et al*. (1998). Chromosome 9 was chosen as a control since it is also a submetacentric 'C' group chromosome of similar size to the X chromosome. As this study aims to validate a FISH method that can easily be incorporated into routine laboratory practice, the use of two centromeric DNA probes was considered desirable to ensure robust fluorescent signals for ease of enumeration (Stupca *et al*., 2005). FISH probe availability, signal strength and financial constraints were also considered when the necessary compromises of FISH probe selection were made.

This method will allow us to clearly define the difference (if any) in results from cultured and un-uncultured techniques. This approach will only ascertain

numerical changes in the X-chromosome (and chromosome 9 as control). This technique will not facilitate investigation of any structural X chromosome abnormality nor any abnormality of other chromosomes.

The method used in this study will allow a relatively non-invasive investigation of the effect of culture on an individual level of X chromosome aneuploidy, and will utilise the same sample type as used in routine cytogenetic practice. Previous studies have compared cultured lymphocytes with fibroblast preparations to determine if the X mosaicism detected in the lymphocytes represents true constitutional mosaicism. Fibroblasts must be obtained from skin biopsies and so represent an invasive test. Furthermore, fibroblasts are not the usual cytogenetic sample type (blood lymphocytes are routinely used) and so changes found in fibroblasts, may not reflect the changes found in routine samples. Buccal cells are easily obtained but have been shown to produce suboptimal FISH signals and are unsuitable for the investigation of mosaicism (Harris *et al*., 1994).

A study of this nature has not been undertaken previously. We will determine if low level X chromosome aneuploidy occurs in the peripheral blood cells of women with proven fertility and establish a "normal range" for this population. If baseline, "normal ranges", of X mosaicism in normally fertile women can be established, findings within or below this range could be considered of no clinical significance in other groups. This study aims to provide a better understanding of the overall significance of low level X mosaicism. Such information will be helpful for accurately counselling women experiencing fertility problems who have had low levels of X chromosome mosaicism documented. We believe this information will assist those counselling women experiencing fertility problems in regards to their likely pregnancy outcomes and the significance of any low level X chromosome mosaicism noted.

In summary, the main objectives of the project were as follows:

1. To determine if true chromosomal mosaicism can be distinguished from artefactual mosaicism (or aneuploidy) by comparing the number of X chromosomes found in peripheral blood samples that have been both cultured and uncultured.

2. To confirm and quantify whether the blood culture process used in cytogenetic testing has a significant effect on X chromosome aneuploidy.

3. To determine the range of low level X chromosome mosaicism present in women with proven fertility using both cultured and uncultured blood samples.

4. To validate the routine use of FISH on uncultured peripheral blood samples to resolve mosaicism.

CHAPTER 2

MATERIALS AND METHODS

Cells from peripheral blood samples of 78 fertile women were harvested from uncultured cell preparations and cultured cells subjected to stimulation with the mitogen phytohaemagglutinin (PHA). Fluorescence in situ hybridisation studies were then performed on cells from both the unculturedand culturedcells using Vysis centromeric-specific probes according to the manufacturer's recommended procedures. Each preparation was then interrogated with fluorescent centromeric-specific probes for the X chromosome and the chromosome 9. The latter was used as an internal hybridisation control. Patient slides were then scored for the number of chromosome X and chromosome 9 signals present in 500 nuclei (1000 nuclei per patient) using fluorescent microscopy. Results were analysed to determine if there is significant variation between the cultured and uncultured blood samples.

2.1 Patient Cohorts

Seventy-eight local West Australian female volunteers participated in the study. Eligible women were aged <40 years and had proven fertility. The median age of women participating in the study was 33 years (range 22-40) with 56/78 (71.8%) aged < 35 years and 22/78 (28.2%) 35 years and older (Appendix 9). Blood samples were collected after obtaining informed consent (Appendices 2, 3, 4 and 5). Proven fertility was defined as at least one natural pregnancy, no history of recurrent miscarriages (defined as three or more consecutive miscarriages), or any fertility treatments (including ovulation tracking, clomiphene citrate, IVF, ICSI, or other invasive treatments) or referrals to a specialist infertility clinic (Appendix 6). Women 40 years and over were excluded in recognition of known age effects on sex chromosome loss (Guttenbach *et al*., 1995; Bukvic *et al*., 2001; Russell *et al*., 2007).

This study was approved by the Curtin University Human Ethics Committee, The Mount Hospital Ethics Committee and the Women's and Children Health Research Institute Ethics Committee at King Edward Memorial Hospital. All participants provided informed consent and were supplied with an information summary for the project (summarised in Appendices 2, 3, 4, 5 and 6).

2.2 Blood Samples

Seven millilitres (7 mLs) of peripheral blood were collected from eligible women into either a sodium heparin or lithium heparin tube as was convenient.

2.3 Lymphocyte Culture

The preparation of cells for chromosomal analysis is sensitive to experimental detail and in some instances the quality of the reagents used. In recognition of this phenomenon, the sources of significant materials together with the compositions of important reagents used in the study have been included.

Cultured preparations from phytohaemagglutinin (PHA) stimulated blood were processed using standard cytogenetic procedures. Participants were allocated to either 72hr or 96hr culture, depending on the day of the week the sample was collected and received. This allowed batching of samples and follows standard practice in cytogenetic laboratories.

In brief, PHA stimulated T lymphocytes are cultured for either 72 or 96 hours and dividing cells are synchronised using thymidine to "block" cells in the "S" phase of the cell cycle. The "block" is then released by the addition of deoxycytidine and the cells harvested 5 hours later as they approach pro-metaphase.

This technique captures extended chromosomes and increases the number of cells arrested at metaphase, hence available for analysis. At pro-metaphase cell division is arrested with colcemid and hypotonic potassium chloride added to swell the cells. The enlarged cells are then preserved with a methanol/acetic acid fixative for microscopic analysis. The detailed experimental procedure is described as follows.

2.3.1 Reagents

Peripheral blood collected in a 7mL sodium or lithium heparin vacutainer tube.

100mL RPMI 1640 w/o L-glutamine (Thermo Trace cat# 11-066) supplemented with

-10mL foetal calf serum (Thermo MultiSer, cat# 15-010-0100V)

- 1mL Penicillin/Streptomycin

- 1mL L-glutamine

- 2mL PHA (Phytohaemagglutinin M Form, Gibco-Invitrogen, Cat # 10576-015)

Thymidine 300µg/mL Sigma, T 9250

Deoxycytidine 4.54 x 10^{-4}g/mL (ICN, Cat # 101484)

KCl Hypotonic (0.057M)

Colcemid 10µg/mL (KaryoMAX® Colcemid® Solution Gibco, Cat # 1512-012)

Methanol/acetic acid fixative (3:1 ratio)

Acetic acid (4% v/v)

Ethanol (for slide cleaning)

2.3.2 Procedure

2.3.2.1 Culture Procedure

0.4mL of well-mixed whole blood was added to supplemented RPMI 1640 medium (10 mL) and incubated at 37°C in 5% CO_2.

At 3pm, the day prior to harvest, 100µL of 30mg/mL thymidine was added to each culture to synchronise the cycling cells in S phase of mitosis.

The following morning, at 8am(i.e. 17hrs after thymidine synchronisation), 100µL of 4.54x10^{-4} g/mL deoxycytidine was added to the cultures to release the mitotic block and allow cells to proceed to prophase of mitosis.

2.3.2.2 Harvesting and Processing Of Cultured Cells

Cells were harvested at 1.00 pm (i.e. 5hrs after deoxycytidine release). All further manipulations of non-fixed cells were performed in a biohazard hood until the cells were fixed.

i. 100µL of colcemid was added to the cell culture and returned to incubate for 15min. This inhibits spindle formation and arrests cells at metaphase.

ii. Cell cultures were then centrifuged at 1300G for 5min and the supernatant aspirated leaving approximately 1mL media above the pellet. Cells were resuspended and 9mL warm hypotonic KCl solution was added. After mixing, the cells were incubated for a further 12min at room temperature and then harvested by centrifugation at 1300G for 5min.

iii. The supernatant was aspirated leaving approximately 1mL hypotonic above the cell pellet. Then resuspended by vortexing the pellet thoroughly. 9mL of 4% acetic acid was added, mixed well and centrifuged 1300G for 5min.

iv. Again supernatant aspirated leaving approximately 1mL of fluid above pellet. Resuspended by vortex. 9mL of 3:1 fixative was then added, mixed and centrifuged as above.

v. The fixation process was repeated two more times, using 5mL of 3:1 fixative.

vi. The majority of the supernatant was aspirated leaving a small amount above the cell pellet. The cell pellet was resuspended and the cell concentration adjusted, if needed, by adding fixative until slightly cloudy.

vii. A drop of cell suspension was placed onto a defined area of an ethanol cleaned slide, allowed to dry and inspected by inverted phase microscopy for cell density prior to fluorescence in situ hybridization.

2.4 Uncultured Lymphocyte Preparation

For uncultured blood preparations, peripheral blood cells were treated directly with a hypotonic solution (0.057M KCl) for 12 minutes, before washing and fixing in methanol: acetic acid (3:1) using a method adapted from Jalal and Law (1997). In brief this method entails the following. Transfer 9mL warm hypotonic solution into 10mL centrifuge tubes and add 0.8mL of blood (≈16 drops) to each tube. Mix well and return to 37°C incubator for 15 min.

Following this step the cells are centrifuged at 1300G for 5 min and the harvested cells then processed as for the cultured cell preparations described above (see 2.3.2.2 Harvesting and processing of cultured cells step iii).

2.5 Fluorescence *in situ* Hybridization (FISH)

Both cultured and uncultured cells for each patient were examined using two-colour FISH analysis with centromere specific enumeration probes (CEP) for chromosomes X and 9 according to manufacturer's instructions (CEP X and CEP

9 Vysis, Inc. Downers Grove, IL, USA)(Horiuchi *et al*., 1997; McInnes *et al*., 1998; Shi & Martin 2000). The same batch and Lot No. for each probe was used in all hybridisations (Stupca *et al*., 2005). Centromeric FISH probes were chosen for enumeration as described by Devi *et al*. (1998). Details of two-colour FISH analysis using a control probe on a chromosome other than the chromosome of interest have been previously described (Horiuchi *et al*., 1997; McInnes *et al*., 1998; Shi & Martin 2000). Details of the procedure used follow.

2.5.1 Reagents

Fixed cytogenetic sample or slide.
DXZ1 CEP Xp11.1-q11.1 SpectrumGreen Satellite DNA probe (Vysis Cat # 05J10-023)

CEP 9 SpectrumOrange probe 9p11-q11 Alpha Satellite DNA (Vysis Cat # 06J36-009)
0.4xSSC/0.3%NP-40 solution
2xSSC/0.1% NP-40 solution
DAPI II Counterstain (Vysis Cat # 06J50-001)
Sterile water (only for dilution)
Vysis hybridisation buffer (only for dilution)

2.5.2 Procedure

2.5.2.1 Probe Preparation

The FISH probe mix consisted of 7 µL of hybridization buffer, 1 µL CEP X DNA probe, 1 µL CEP 9 DNA probe and 1 µL purified H2O.

2.5.2.2 Co-Denaturation and Hybridisation

2µL of probe mix was applied to each target area on the patients slide, then coversliped and sealed with rubber cement.

The patient sample and the FISH probe were then co-denatured at 74+/-1ºC on a hotplate for 5mins.

Slides then hybridised overnight at 37°C (minimum 4hrs). If necessary, slides were left to hybridise over the weekend.

The rubber cement seal and the coverslip were removed from the slide, the slide was then washed in a series of solutions to remove unhybridised FISH probe. These washes consisted of 0.4xSSC/0.3%NP-40 wash solution at 73+/-1ºC for 2 minutes. Then, 2xSSC/0.1% NP-40 at room temperature for 30 seconds to 1 minute. The slides were then dried in darkness, followed by counterstaining with DAPI II and coversliped. Slides were stored at - 20°C in darkness.

2.6 Cell Counts and FISH Signal Enumeration

Samples were examined as de-identified paired observations; 500 cells from both the cultured and uncultured preparations were scored providing a total of 1000 cells per case. The study could not be blinded because of clear differences in the appearance of uncultured (direct) and cultured preparations. The uncultured (direct) preparations showed smaller compact interphase nuclei. The cultured preparations demonstrated large interphase nuclei and the presence of metaphase cells (Figure 2.1 and Figure 2.2).

Only nuclei that were not overlapping or adjoining other cells and had two signals for the internal chromosome 9 control were scored. Following the work by Guttenbach *et al*., (1995), if nuclei hybridised with the X centromeric probe showed only one obvious signal, that was larger than the signals in neighbouring cells, then the one larger signal was regarded as two fused signals. The cell was considered to be disomic however it was not included in the analysis. Conversely, nuclei showing 2 signals, one of which was a doublet, were scored as disomic (Edmonds et al., 1982). Nuclei without two signals for the X chromosome were classed as aneuploidy (Figure 2.3 and Figure 2.4). These included cells with 1, 3, 4, or 5 X centromeric signals. This is consistent with the scoring system used by Horiuchi *et al*., 1997.

Five hundred (500) cells counted per preparation was chosen since it allows for a background rate of aneuploidy of less than 1% (±0.5%) with 99% confidence limits. This figure is the same as that proposed previously by Tonecheva *et al*., (1994) and is in agreement with the sample size obtained using the methods of Desu et al. 1990 and Machin et al (1997). To achieve a confidence level of 99%, it was determined that a minimum of 421 cells be counted. Analysis of 500 cells allows detection of a background rate of aneuploidy from as low as 0.4% to 1% in cells.

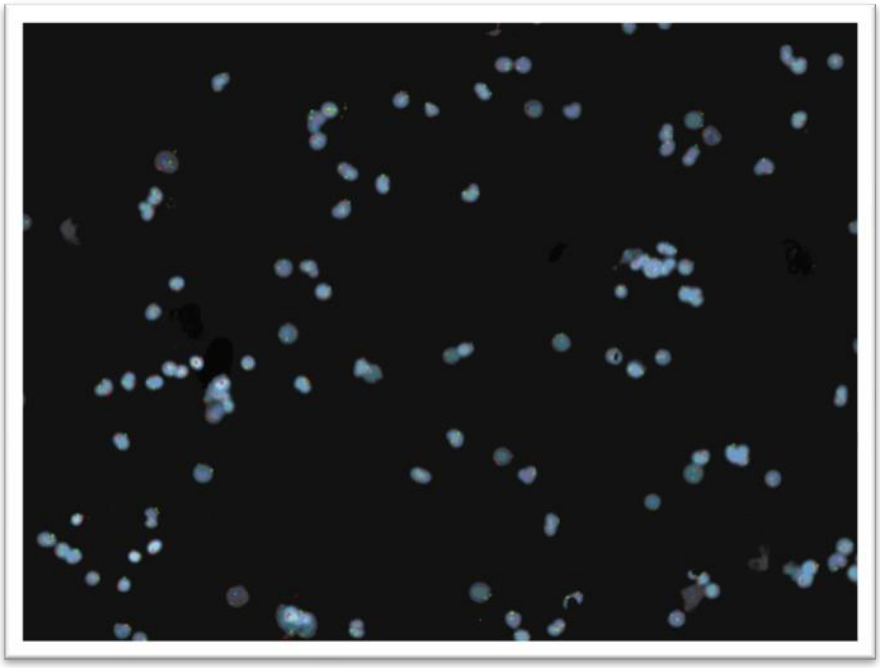


Figure 2.1: Interphase nuclei from uncultured preparation.

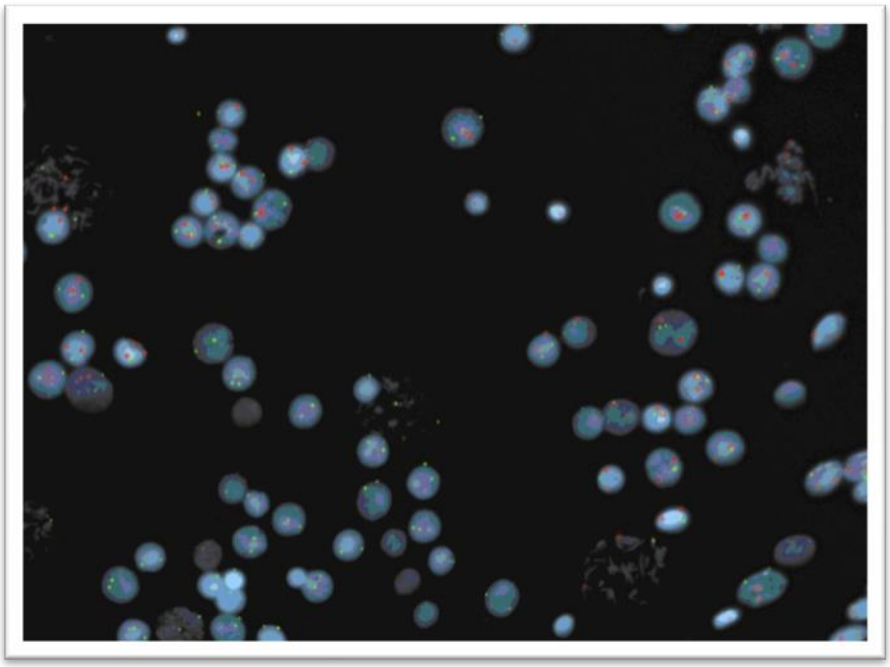


Figure 2.2: Cells from cultured preparation showing interphase nuclei and metaphase cells.

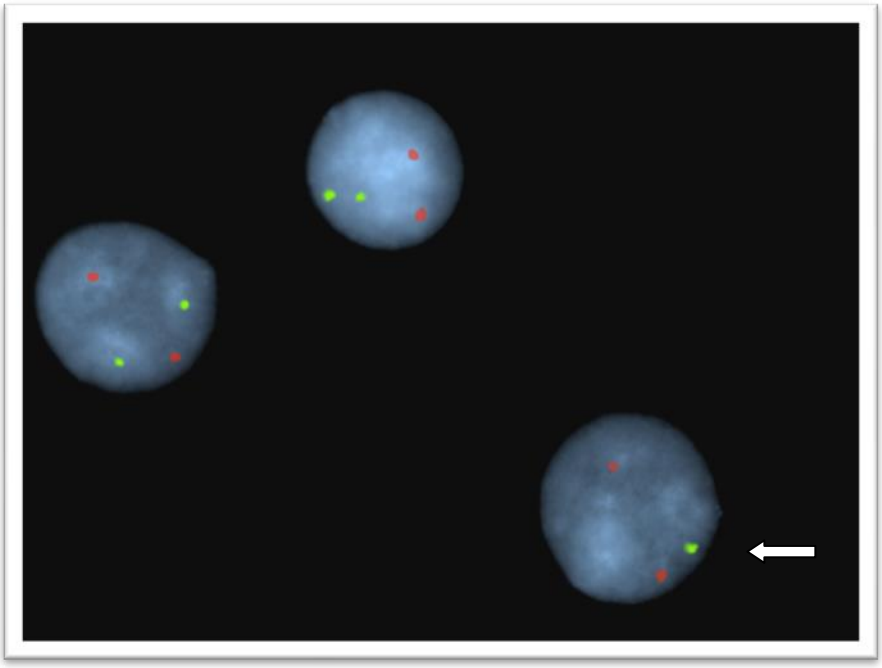


Figure 2.3: Interphase nuclei with 1 x chromosome X signal (green with arrow) and 2 x chromosome 9 signals (red).

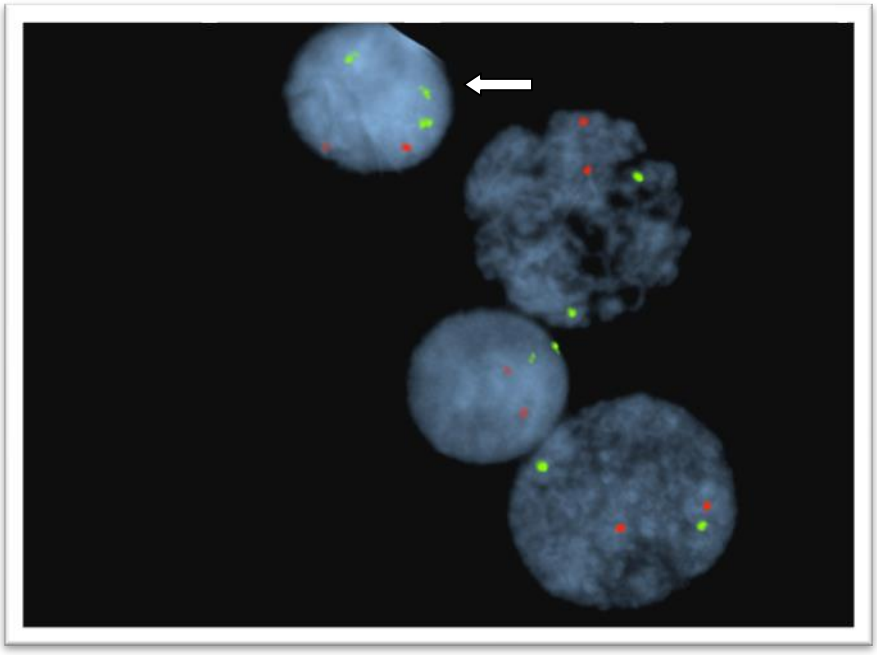


Figure 2.4: Interphase nuclei with 3 x chromosome X signal (green with arrow) and 2 x chromosome 9 signals (red).

2.7 Data and Statistical Analysis

The statistical analyses included in this project summarised continuous data using medians and percentiles, or means and standard deviations. Categorical data were summarised using frequency distributions. Univariate comparisons between uncultured and cultured cells were conducted using the Mann–Whitney test for independent samples and the Wilcoxon Signed Ranks test for paired samples. Poisson regression analysis for paired samples was performed and covariate effects were summarised using rate ratios (RR) and their 95% confidence intervals (CI). Statistical analyses were completed using the Statistical Package for the Social Sciences Application (SPSS - for Windows version 11.0, Chicago, IL, USA) and Excel (Microsoft) together with LogXact (LogXact version 5; Cytel, Cambridge, MA, USA) All p values were two-sided and p-values <0.05 were considered statistically significant. Where appropriate regression analyses were performed using JMP version 10 published by the SAS Institute Inc., (USA).

The sample size of 78 paired samples was chosen to allow detection of a rate ratio of 2.0 for X aneuploid cells associated with the 'cultured' cells with ≥80% power and 5% significance level using Poisson regression. This sample size was estimated using the Power and Sample Size for Windows software application (PASS 2002, Kaysville Utah), assuming a minimum mean background rate of aneuploid cells between 0.4% and 1% and deemed appropriate considering the previously reported rates of X aneuploidy of 3.2% to 11.6% reported by several earlier reports (Meschede *et al*., 1998; Scholtes *et al*., 1998; Peschka *et al*., 1999; Sonntag *et al*., 2001; Morel *et al*., 2002; Morel *et al*., 2004).
The number of cells to be counted was calculated using assumptions that the background rate of aneuploidy may not exceed 1%, and that the estimation of this rate was to attain +/- 0.5% rate with 99% confidence. Tonecheva *et al*., (1994) have reported that the analysis of 500 cells is needed to detect low level X chromosome aneuploidy at a level of 1% abnormal clones, if the background level of same aberrations in controls is 0.1%. It was estimated that to attain a confidence level of 99% 421 cells were required. However for each sample 500 cells were counted to address the possibility that the assumed background rate of 1% was lower. Analysis of 500 cells allows detection of a background rate of aneuploidy from as low as 0.4% to 1%.

CHAPTER 3

X CHROMOSOME ANEUPLOIDY IN FERTILE WOMEN

Background levels of X chromosome aneuploidy are reported in a cohort of 78 West Australian women using both cultured and uncultured peripheral blood. Only women with demonstrated fertility were included. All women were between the age of 22-40 years, at least primigravida, and without known fertility problems.

3.1 Introduction

Routine chromosome analysis (karyotyping) plays an important role in modern medicine. However, it is an expensive laboratory procedure which currently attracts an Australian Government Medicare benefit schedule (MBS) fee of $358.95 (MBS Online; Item 73289) and its use is usually restricted to those instances where the predictive value of the procedure offers important clinical information. There is therefore a lack of cytogenetic data for women without known fertility problems. In this study blood samples from 78 fertile women (as described in Chapter 2) were subjected to cytogenetic analysis using FISH specifically to identify aneuploidy of the X chromosome. All subjects were aged between 22-40 years and had given informed consent for the study.

The study entailed examining nuclei for X aneuploidy by FISH from cells that were tested both with and without PHA stimulation. The details of the two culture methods are important and these are described in Chapter 2. The two cohorts of cells are described herein as "cultured cells" and "uncultured cells".

A total of 78,000 interphase nuclei from the 78 participants were analysed by FISH for X aneuploidy. As described in Chapter 2, samples were examined as anonymous paired observations; 500 cells from each of the cultured and uncultured preparations were scored for each patient providing a total of 1000 cells per case.

Cells were examined using two-colour FISH analysis with centromere specific enumeration probes (CEP) for chromosomes X and 9, as described in Chapter 2. Only nuclei that were not overlapping, or adjoining other cells and with two

signals for the internal chromosome 9 control, were scored. Nuclei without two signals for the X chromosome were classed as aneuploid.

3.2 Results

Interpretation of the results reported herein requires approximately equal efficiencies of hybridisation of the X chromosome and chromosome 9 FISH probes used. Both probes were equally sensitive showing 98.8% binding in the nuclei of uncultured cells and 99.4% binding in the nuclei of stimulated cells. This falls within the range reported in a large multicentre study by Dewald et al. (1998). The hybridisation rate between cultured and uncultured cell preparations was not significantly different ($p \geq 0.066$). These data are included in Appendices 7 and 8.

3.2.1 X Chromosome Aneuploidy in Uncultured and Cultured Cells

The effect of cultured and uncultured cell preparation on the proportion of X chromosome FISH signals per cell was analysed. A general overview of the differences between the two methods is summarised in Table 3.1 and diagrammatically in Figure 3.1. As expected, X chromosome aneuploidy was found in higher levels in the cultured cell preparations. The mean rate of aneuploidy was significantly greater in cultured (2.1%) compared to uncultured cell (0.9%) preparations ($p < 0.001$). The mean number of cells with aneuploid X chromosome signals in the uncultured group was 4.5 (95% CI 3.7-5.4), and in the cultured group was 10.6 (95% CI 9.5-11.7). The range was 0.0% - 3.8 % for uncultured samples and 0.2% - 4.6% for cultured samples again this difference was significant ($p< 0.001$) (Table 3.1). Cultured cells showed a relative rate of 2.3 (95% CI 2.1-2.6) for producing X aneuploid cells compared with uncultured preparations.

Table 3.1: Comparison of cells showing X chromosome aneuploidy in 'Uncultured' and 'Cultured' peripheral blood samples for 78 fertile women.

Mean Aneuploidy / 500 cells		**Uncultured#**	**Cultured#**	**p-value**
All n = 78	n	**4.5** (Range 0-19) (95% CI* 3.7-5.4)	**10.6** (Range 1-23) (95% CI 9.5-11.7)	<0.001
	%	**0.91** (Range 0-3.8)	**2.1** (Range 0-4.6)	

Means and * 95% confidence intervals.

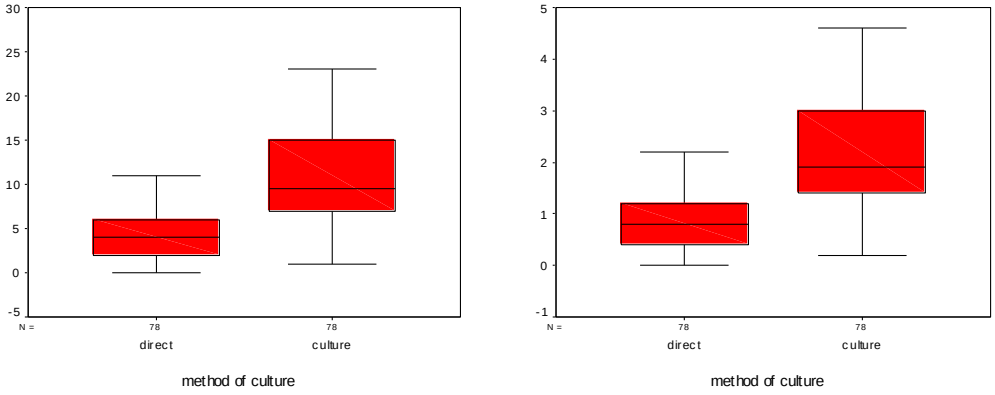


Figure 3.1: Comparison of Mean and Ranges of cells showing X centromere signal aneuploidy in 'Uncultured' and 'Cultured' blood samples. ***A.*** *Mean number of X aberrations versus method.* ***B.****Percent of aberrations versus method.*

3.2.2 Distribution of X Chromosome Aberrations

The number of X chromosome FISH signals lost or gained for each sample analysed was recorded in this study. Chromosome abnormalities ranged from zero to 23 per 500 nuclei per individual. X chromosome loss occurred more frequently than gain. The frequency of X chromosome loss was 0.8 % in uncultured cells and 1.56% in cultured cells (Chi Square = 7.5; $p < 0.006$). This compared with triple X detected in 0.1% of uncultured cells and 0.54% in cultured (Chi Square = 560; $p < 0.0001$). Cells with four or five FISH signals for the X chromosome were only detected in cultured cells at extremely low levels

(Table 3.2). The frequency of distribution of chromosome abnormalities per 500 nuclei per individual is shown in Figure 3.2.

Table 3.2: Frequencies of X chromosome aneuploidy from 'Uncultured 'and 'Cultured' cell preparations each in 39000 interphase cells.

Chromosome Count	X	XX	XXX	XXXX	XXXXX	Total
Uncultured n	305	38,647	48	0	0	39,000
Uncultured %	0.80	99.10	0.1	0.0	0.0	100%
Cultured n	604	38,175	212	8	1	39,000
Cultured %	1.56	97.88	0.54	0.02	0.0	100%

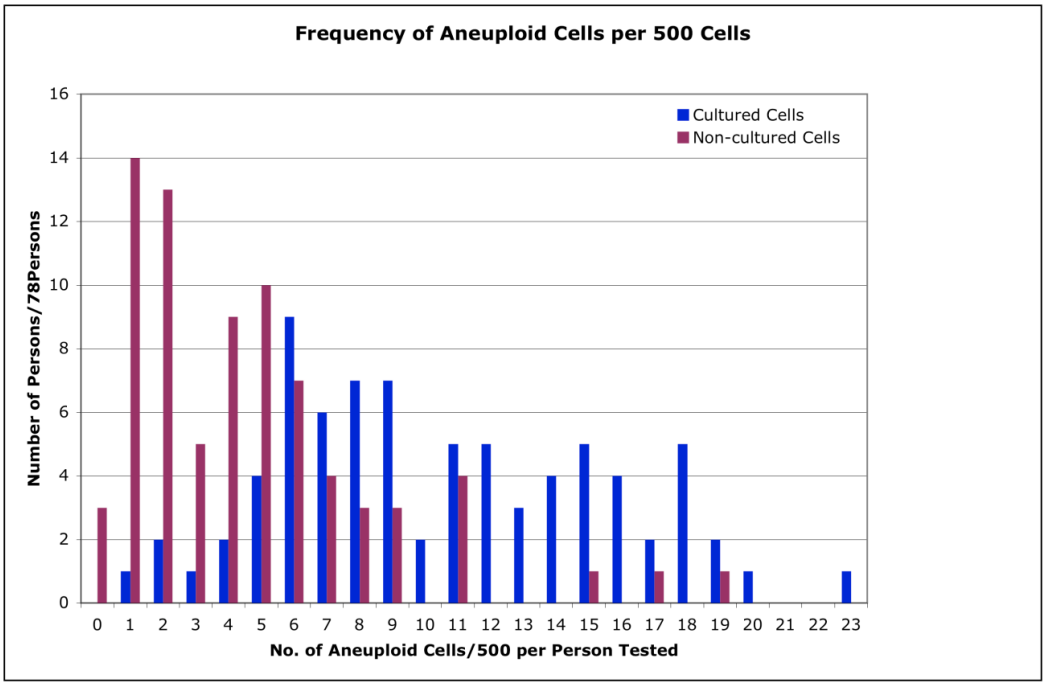


Figure 3.2: Frequency distribution of chromosome abnormalities per 500 nuclei per individual in 'Uncultured' and 'Cultured' cell preparations.

Comparison of median values for differences between cultured and non-cultured cells for each of the 78 women tested also demonstrated the increased frequency of X chromosome FISH signal loss versus gain (Table 3.3.). The median value for X chromosome FISH signal loss in uncultured cells was 4 which matched the overall aneuploid cell signal median. The median value of cells with one FISH signal for the X chromosome in cultured cells was 7, while the median value for all aneuploid signals was 9.5.

It is clear that more abnormalities were seen in cultured cells relative to uncultured cells, but importantly, 75 of the 78 women tested showed some abnormality irrespective of the method used. The raw data for each individual patient's FISH analysis and resultant level of chromosome X aneuploidy in both uncultured and cultured cells are reported in Appendices 9 and 10.

Table 3.3: Median values for chromosome counts between cultured and unculturedcells for each of the 78 women tested.

Method	Cells with 2X/500 nuclei	Median Abnormalities/ sample/500 nuclei	Cells with 1X/T	Cells with 3X/T	Cells with 4X/T	Cells with 5X/T
Uncultured	496	4	4	1	0	0
Cultured	490.5	9.5	7	3	1	1

3.3 Discussion

As far as is known, this study is the first to provide X chromosome aneuploidy frequency data from fertile women of reproductive age, using paired samples of cells both with and without stimulation with PHA. The availability of data on the rate of X chromosome mosaicism or aneuploidy on women with normal fertility is limited. A widely referenced study by Horsman *et al*., (1987) reported <10% X chromosome mosaicism for a cohort of 'fertile' women. However the criteria used for 'fertility' in this report is ambiguous. Tonecheva *et al.,* (1994) found frequencies of 0.12% monosomy X and 0.05% trisomy X in a very small control group of 6 fertile women. A larger study on a control group of 174 age matched

women found a frequency of 6.9% for monosomy X and 2.9% for trisomy X (Nowinski *et al*., 1990). Morrel *et al.,* (2002) found X chromosome loss or gain in 22% of their normal control group. Unlike the present study the fertility status was not clearly defined or reported for either of the groups used in the studies by Nowinski *et al.,* (1990) and Morrel *et al.,* (2002).

Numerous studies have reported low level X chromosome mosaicism in women referred for fertility treatments. Horsman *et al*., (1987) found low level X chromosome mosaicism (2-10%) occurred in 16% (17/103) of infertile women studied. Other studies have found lower rates of X chromosome mosaicism in women presenting for fertility treatments with rates of 4.6%, 7.2%, 3.3%, 3.2%, 9.6% and 10.5% reported by Meschede *et al*., (1998); Scholtes et al., (1998); Peschka *et al*., (1999); Sonntag *et al*., (2001); Morrel *et al*., (2002) and Morrel *et al*., (2004) respectively. A review of literature by Morel *et al*., (2004) reduces the frequency of numerical sex chromosome abnormalities (usually low level mosaicism) detected in women presenting for ICSI to 4.44%.

This study demonstrates unequivocally that the incidence of X chromosome aneuploidy increases significantly following cell culture stimulation with the mitogen PHA. As shown in Tables 3.1, 3.2 and 3.3 the presence of PHA in cell cultures on average doubles the frequency of X chromosome aneuploidy when compared with uncultured cells. The comparison of the uncultured and cultured paired samples proves that this increase in X chromosome aneuploidy can in fact be attributed to cultural artefact.

In this study the rate of X chromosome aneuploidy was <5% for all women irrespective of the method used for cell preparation and is consistent with the earlier findings of Guttenbach *et al*., (1995) who demonstrated that sex chromosome loss was only significant beyond reproductive age (>51 years). This study supports the findings of Peschka *et al*., (1999) who define low level mosaicism as <6% rather than the cut-off of <10% commonly used in routine diagnostic cytogenetic laboratory's. This limit resulted from the work of Horsman *et al.,* (1987) and is referred to in Gardner and Sutherland (2004) and is suggested to be without reproductive significance in the phenotypically normal woman. However, as stated previously, the fertility status of the control group of women used in this work is ambiguous.

The loss of chromosome X FISH signal was detected more frequently than gain in both the cultured and cultured cell preparations. This suggests either a selective advantage of monosomic cells or a disadvantage of trisomic cells or that losses result from a technical bias (Richard *et al*., 1993). The inactive X chromosome has already been shown to have an increased incidence of micronucleation (Catalan *et al*., 1998; Catalan *et al*., 2000; Bukvic *et al*., 2001; Wodjda and Witt, 2003; Wodjda *et al*., 2006). Other proposed mechanisms for the increased incidence of X chromosome loss include premature centromere division and subsequent non-disjunction of the X chromosome (Fitzgerald, 1975). Abruzzo *et al*., (1985) have shown that the inactive X chromosome is missing in most monosomy X cells. Guttenbach *et al*., (1995) support this finding with the observation that premature centromere division of the X chromosome has a lower incidence in males and it therefore likely that it is the inactive X chromosome that is preferentially prone to premature centromere division.

Finally, X chromosome loss has previously been shown to be affected by the culture process itself, being significantly more frequent in 72 hour than in 48 hour cultures (Richard *et al*., 1993). The data from this study is consistent with the findings of Richard *et al*. (1993) since a significant difference was found in the rate of X chromosome loss in the cultured cells compared to the uncultured cells.

Although not a main focus of this study, it was observed that chromosome 9 aneuploidy occurred very much less frequently than did X chromosome aneuploidy. Breakage of the chromosome 9 centromeric heterochromatin detected by the classical alpha satellite DNA probe as reported by Edmonds *et al*. (1982) was not observed in this study. This may be attributed to the robustness of the Vysis DNA probe or the lack of clastogenic agents used. Since chromosome 9 is of similar size to chromosome X and are both submetacentric, it would seem that chromosome X has a greater predisposition to aneuploidy in cell culture than chromosome 9 and this may be accounted for by the mechanisms previously discussed. This *in vitro* observation is reflected in the relative proportions of chromosome X and chromosome 9 aneuploidy (possibly all autosomal aneuploidy) observed in routine cytogenetic analysis that in turn reflects *in vivo* frequencies of aneuploidy associated with disease (Guttenbach *et al*., 1995). These findings correlate with earlier studies by Ford and Russell, (1985); Nowinski *et al*. (1990); Richard *et al*. (1993); Guttenbach *et al*. (1995); that showed no correlation with age and autosome loss.

These results support the further investigation of X chromosome aneuploidy of greater than 5% discovered during routine karyotyping by using FISH on uncultured cells. In doing so this method may provide results that will be immediately useful in either attributing low level X chromosome aneuploidy to cultural artefact or the presence of true constitutional mosaicism.

CHAPTER 4

SUBJECT AGE, CULTURE TIME, GRAVIDITY ANDPARITY

During the course of this project, data was collected on the effect of time in mitogen stimulated cell culture and subject age on levels of X chromosome aneuploidy in peripheral blood cell cultures. Data was also collected on the subject's gravida and para status. Aneuploidy rates for 72 and 96 hour cultures were compared with no significant effects being observed. Further, a small increase in aneuploidy was observed in women 35 years and older relative to women less than 35 years. These results assist in the interpretation of aneuploidy observed in cytogenetic tests for fertility.

4.1 Introduction

It has been suspected for many years that observed rates of some chromosomal abnormalities are influenced by variation in the culture methods used. Extended culture times result in more abnormalities (Rooney and Czepulkowski, 1992). Because of this, in many laboratories it is believed that a 72 hour culture is better than a 96 hour culture. However, management of staffing levels, financial budget and cytogenetic testing timeframes may benefit from a 96 hour culture period relative to the more frequently employed 72 hour cultures. Consequently, X chromosome aneuploidy rates were determined in both 72 and 96 hour cultures.

Current cytogenetic practice utilises mitogen (PHA) stimulated peripheral blood T-lymphocytes to increase the number of cells available for analysis after 72 -96 hours in cell culture (Hungerford *et al*., 1959). However PHA stimulation is also known to increase the number of aneuploid cells in the final cell preparation (Rooney and Czepulkowski, 1992). It is recognised that growing cells in culture may alter the proportions of abnormal cells present (Rooney and Czepulkowski, 1992). In carrying out chromosomal analysis, the cells examined have undergone transformation and growth *invitro* and these culturing processes themselves may change the proportions of abnormal cells examined (Rooney

and Czepulkowski, 1992). "Cultural artefacts" is the term used to describe findings considered generated merely from culturing processes.
The pathological significance of aneuploidy observed in the cultured cells, but not otherwise detected, of an individual is therefore controversial. For this reason we have used an uncultured preparation of peripheral blood to compare with a blood sample cultured as per standard cytogenetic techniques.

Furthermore since it is known that X chromosome loss is more frequentlyobserved in women of increasing age (Guttenbach *et al*., 1995; Bukic *et al*., 2001; Russell *et al*., 2007), aneuploidy rates in the women used in this study were investigated as a function of age. Women over 40 years were excluded in recognition of the known age effect on sex chromosome loss (Guttenbach *et al.*, 1995; Bukvic *et al.*, 2001; Russell *et al*., 2007).

The cohort used in this study was sub-divided into subjects aged 35 years and older and subjects less than 35 years of age. The cut off of 35 years is commonly referred to as 'advanced maternal age' and is used to assess associated obstetric risk factors (Ales *et al*., 1990; Cleary-Goldman *et al*., 2005; Tough *et al*., 2007; Wang *et al*., 2011; Bayarampour *et al.*, 2012). These include a higher risk of miscarriage, chromosomal abnormalities, gestational diabetes, hypertension, placenta previa, preterm birth, low birth weight, caesarean delivery and hospitalization during pregnancy. (Ales *et al*., 1990; Cleary-Goldman *et al*., 2005; Tough *et al*., 2007; Wang *et al.*, 2011; SOGC, 2012).

During volunteer recruitment, obstetric history was assessed to ensure the cohort all met the criteria established for this study of confirmed fertility (Appendix 6). Subsequently, data on the gravidity and parity of the volunteers was collated.

4.2 Results

4.2.1 Maternal Age and PHA Stimulation

The effect of maternal age and cell preparation (uncultured versus cultured) on the proportion of X chromosome aneuploid cells was analysed. The results are depicted in Figure 4.1, which shows that for this cohort of 78 women cell culture causes an increase in the proportion of X chromosome aneuploidy, especially for

older women. Linear regression analysis using the JMP software application (version 10, 2012) revealed (R^2 = 0.10978) that in the presence of cell culture ≈11% of the total variance in X chromosome aneuploidy is accounted for by maternal age. In the absence of cell culture, however, no age-dependent component of variance in X aneuploidy is detectable. The combined effect of cell culture and age of the women tested is highly significant ($p \leq 0.003$) and contrasts to the constancy of the proportion of X chromosome aneuploidy in uncultured cells for all 78 women in the cohort tested ($p \geq 0.878$). These data are summarised in Figure 4.1 and Table 4.1.

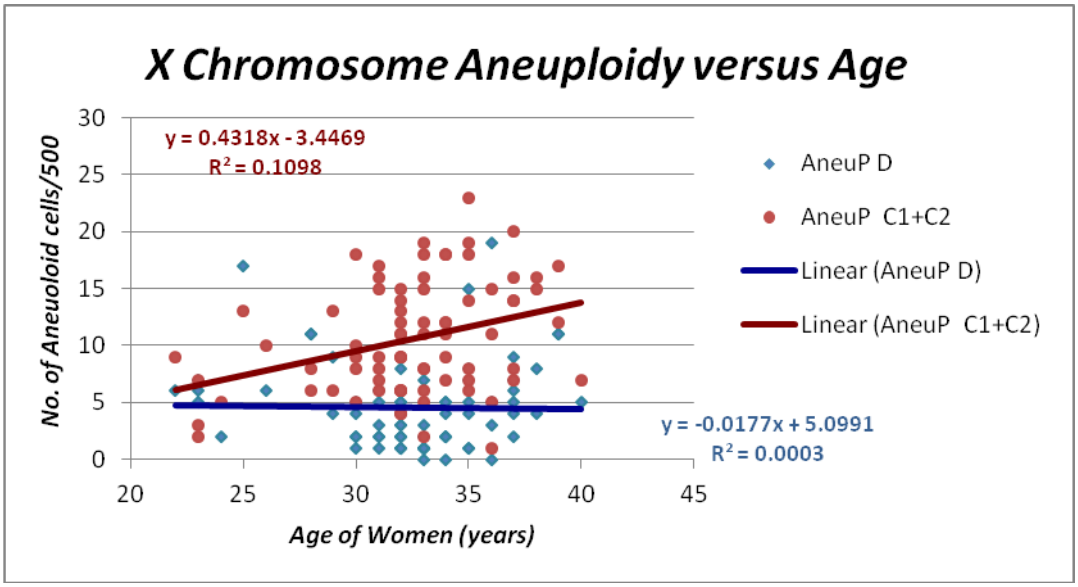


Figure 4.1: The proportion of aneuploidy X chromosomes/500 cells for uncultured and cultured cells (72+96 hr cultures), together with linear regression lines and equations for each class. Blue tones depict uncultured cells (labelled AneuP D in legend) while red tones are for cultured cells (labelled AneuP C1+C2).

By comparing two groups of women - those aged less than 35 years (n=56) and those aged 35 years and above (n=22), it was shown that the increased X chromosome aneuploidy resulting from cultured cells relative to uncultured cells was highly significant for both subgroups as well as the combined cohort. These results are summarised in Table 4.2.

Table 4.1: Analysis of Variance of X Chromosome Aneuploidy (Cultured and Uncultured Cells) versus Age – Linear regression model on 78 women aged 22 – 40 years.

Source	Regression	DF	Sum of Squares	Mean Square	F Ratio
Cultured Cells		1	205.4082	205.408	F = 9.3724
	Error	76	1665.6303	21.916	
	C. Total	77	1871.0385		**p = 0.003**
Uncultured Cells		1	0.3434	0.3434	F = 0.0239
	Error	76	1093.1053	14.3830	
	C. Total	77	1093.4487		**p = 0.8776**

Table 4.2: Comparison of cells showing X chromosome aneuploidy in uncultured versus cultured peripheral blood lymphocyte samples for women less than 35 years of age and women ≥ 35 years.

Mean Aneuploidy / 500 cells		Uncultured#	Cultured#	p-value
All n = 78	n	4.5 (R 0-19)	10.6 (R 1-23)	<0.001
	%	0.91 (R 0-3.8)	2.1 (R 0-4.6)	
Age <35 years n = 56	n	3.8 (R 0-17)	6.4 (R 0-19)	<0.001
	%	0.76 (R 0-3.4)	1.3 (R 0-3.8)	
Age ≥35 years n = 22	n	9.9 (R 2-19)	12.4 (R 1-23)	0.001
	%	2.0 (R 0.4-3.8)	2.5 (R 0.2-4.6)	

medians, and ranges (R).

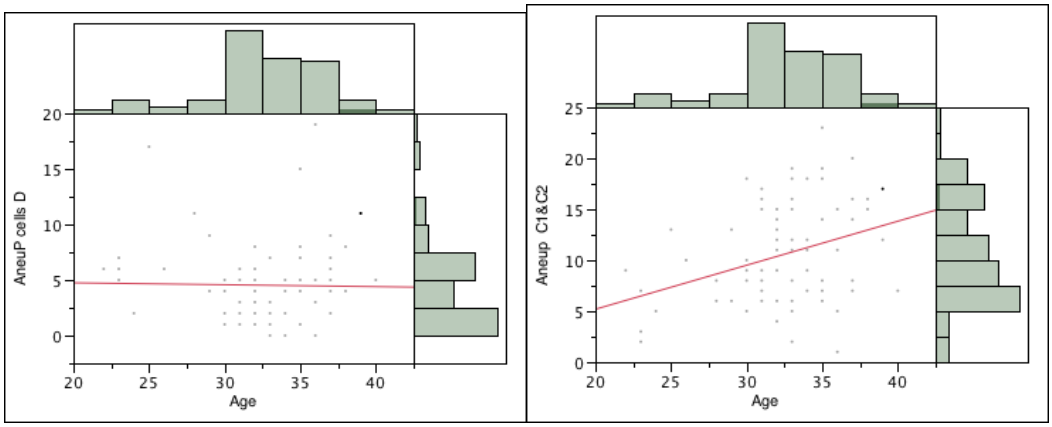
The lowest rate of aneuploidy was found in the younger women (< 35 yrs) when uncultured cell preparation was used and a relative rate of 1.0 was assigned to this group. Cultured cell preparations from younger women (< 35 yrs), cultured cell preparations from older women (≥ 35 yrs) and uncultured cell preparations from older women (≥ 35 yrs); all showed a significant difference ($p < 0.001$) in X

chromosome aneuploidy when compared to this rate. The relative rates and 95% confidence intervals for the other groups and culture conditions are summarised in Table 4.3. The highest rate of aneuploidy occurred in cultured cells for women aged 35 years and over with a relative rate of 3.26 (95% CI 2.73 - 3.90).

The distributions of age of women tested and the rate of aneuploidy for uncultured cells and cells cultured for 72 and 96 hours have been summarised in the figures below (Figure 4.2). It can be seen from the regression lines that the rate of aneuploidy for uncultured cells is not correlated with age, whereas there is a distinct positive correlation ($p < 0.003$) for cells cultured with PHA.

Table 4.3: Relative rates of X chromosome aneuploidy signal in women with a combination of culture method and age.

Method	Relative Rate	95% CI	p-value
Uncultured preparation in Women < 35	1.00		
Uncultured preparation in Women ≥ 35	1.67	1.35 – 2.07	<0.001
Cultured preparation in Women < 35	2.59	2.21 – 3.03	<0.001
Cultured preparation in Women ≥ 35	3.26	2.73 – 3.90	<0.001



A **B**

Figure 4.2: Rate of aneuploidy X chromosomes/500 cells for uncultured (A) and cultured cells (B), together with linear regression lines (red) and histograms showing the distributions of both variable. Uncultured cells labelled AneuP D in legend while cultured cells (labelled AneuP C1+C2=72hr+96hr). The regression line for rate of aneuploidy versus age is shown in red.

4.2.2 Length of Culture Time

The effect of time spent in culture and cell preparation (cultured or uncultured) on X chromosome number was analysed by dividing participants into two groups – 72hr (n=53) or 96hr (n=25) culture. Participants were allocated to either 72hr or 96hr culture, depending on the day of the week the sample was collected and received into the laboratory. This allowed batching of samples and follows standard practice in cytogenetic laboratories.

It was unexpectedly observed that cases randomly allocated to the 72hr culture group had significantly more X chromosome aneuploidy than those randomly allocated to the 96hr culture group (11.1 median aneuploidy compared to 9.4 median aneuploidy, p= 0.004) using Poisson regression analysis (Table 4.4). However, after adjustment for the number of aneuploid cells found using the uncultured method for each individual (median cell numbers 5.2 compared with 3.1 for uncultured method corresponding to 72hr and 96hr cultures respectively, p=0.018), no significant difference was found between the number of cells with X aneuploidy obtained at 72 hours compared to 96 hours (p=0.150). The mean and median ages of the women in each group were 33 and 32 years respectively.

Table 4.4: Comparison of cells showing X chromosome aneuploidy after '72 hrs' or '96 hrs' in culture.

Mean Aneuploidy / 500 cells		**72 hour culture# (n=53)**	**96 hour culture# (n=25)**	**p-value**
Random Allocation	n	11.11 (R 1-22)	9.44 (R 3-19)	0.004
	%	2.22(R 0.2-4.4)	1.89 (R 0.6-3.8)	
Individually Adjusted	n	5.21 (R 0-19)	3.08 R 1-8)	0.018
	%	1.04 (R 0-3.8)	0.62(R 0.2-1.6)	

medians, and ranges (R).

4.2.3 Maternal Age and Length of Culture Time

The effects of time in PHA stimulated cell culture (72 versus 96 hours) and age in women was investigated using linear regression analysis with the JMP application. These data are shown in figure 4.3below. It can be seen that the rate of X chromosome aneuploidy increased with the age of the women for cultures of both 72 and 96 hours duration. The effect was more pronounced with 96 hr cultures (R^2 =0.15 versus 0.07 for the 72 hr cultures). These data indicate that blood lymphocytes from older women are more susceptible to X chromosome aneuploidy than lymphocytes from younger women for both 72 and 96 hr cultures. Analysis of variance confirmed that the effect observed was just significant for the 72 hr cultures (p = 0.044, N = 53) and not significant for the 96 hr cultures (p = 0.055, N =25).

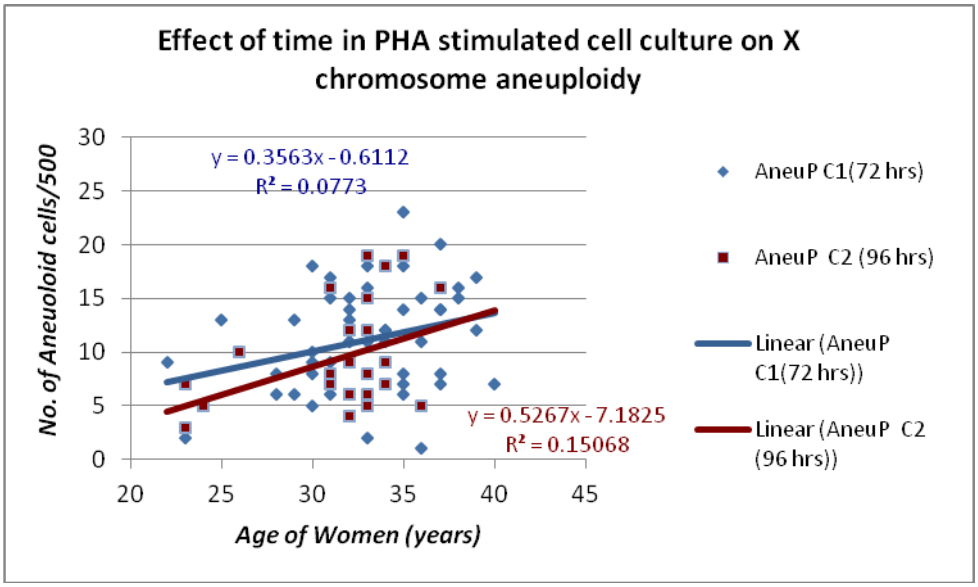


Figure 4.3: The proportion of aneuploidy X chromosomes/500 cells for cultured cells, together with linear regression lines and equations for each class. Blue tones are for 72hr cell cultures (labelled AneuP C1 in legend) while redtones depict 96hr cell cultures (labelled AneuP C2).

4.2.4 Effect Of Anecdotal Number Of Pregnancies (Gravidity) and Number of Children (Parity) Versus Age of Women Tested.

The numbers of pregnancies recalled by each woman, as well as the number of children borne by each woman were recorded during this study. Miscarriage and termination were explored sensitively. However, the number of pregnancies is an anecdotal figure and almost certainly an underestimate since memory is often faulty and an early miscarriage (*blighted ovum*) may not be recognised (Miller *et al*., 1980).Nor may some volunteers wish to disclose details of miscarriage and termination. In contrast the number of children borne by each woman is an accurate number. These data are presented in Figure 4.4.

Neither the number of recalled pregnancies, nor the number of children for each woman correlated with age. When number of recalled pregnancies versus number of children was plotted, a significant correlation ($p \leq 0.001$) was observed ($R^2 = 0.439$), with ≈44% of the variation in number of recalled pregnancies being associated with the number of children borne by each woman (see Figure 4.4). This positive association is expected despite the potential errors in the recorded number of recalled pregnancies. When gravidity and parity were

analysed irrespective of age (Figure 4.5) the slope of the regression line was close to 1.0 (0.94) as expected; since the number of recalled pregnancies exceeds the number of children born to each women.

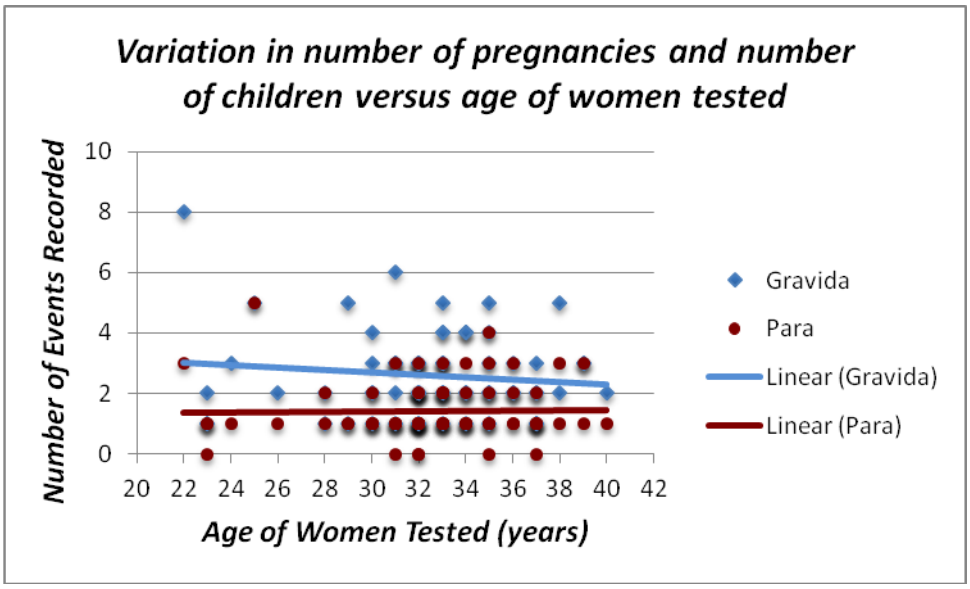


Figure 4.4: The gravidity, parity and age of women tested, together with linear regression lines and equations for each class. Red tones are for parity while blue tones depict parity.

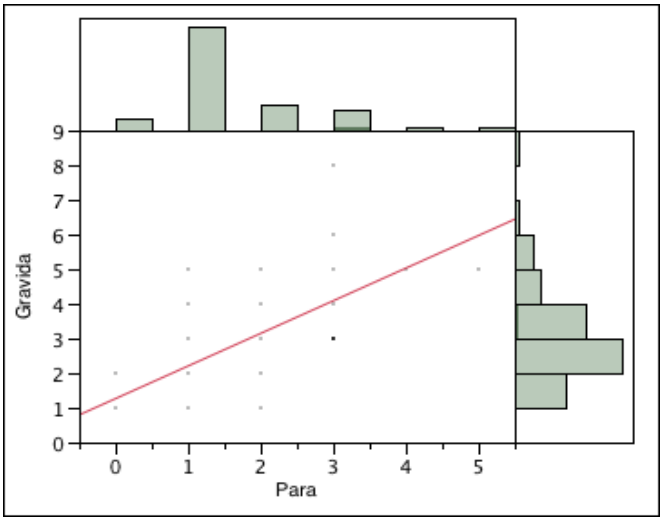


*Figure 4.5: Regression of Gravida against parity per woman in the cohort tested together with the distribution for each variable. The regression line is Gravida = 0.94*Parity +1.27.*

4.3. Discussion

Previously reported effects of age on the rate of X chromosome aneuploidy found during karyotyping (Nowinski *et al*., 1990; Guttenbach *et al*., 1995; Russell *et al*., 2007) were confirmed in this study. With a higher rate of aneuploidy noted in women $\geq$35 years compared to younger women. Further study of fertile women over the age of 40 may be helpful to assess the rate of aneuploidy in this group.

Cultured cells showed an increase in the rate of X chromosome aneuploidy that correlated positively with increased age of the women for cultures of both 72 and 96 hours duration. This finding supports that of earlier work by Nowinski *et al*., 1990; Guttenbach *et al*., 1995; Russell *et al*., 2007. These data indicate that blood lymphocytes from older women are more susceptible to X chromosome aneuploidy than lymphocytes from younger women for both 72 and 96 hr cultures. Obviously, we can't comment on any non-significant trends observed in the data for the 72 and 96 hour culture experiments. Of course, if these experiments were repeated with a larger sample of subjects, a greater statistical power would be obtained.

Interestingly, increasing age did not affect the rate of detection of X chromosome aneuploidy in the uncultured preparations. Linear regression analysis using the JMP software application showed only 0.03% variability (R^2 = 0.0003) in X chromosome aneuploidy with age in the uncultured cell preparations.

This result shows clearly that the higher rates of aneuploidy in cultured cell preparations can be partially explained by cultural artefact rather than solely on the age of the women tested. This finding further supports the use of the uncultured cell preparation technique to help elucidate the interpretation of low level X chromosome mosaicism.

Initial comparison of X chromosome aneuploidy between the 72 and 96 hour cultures displayed a significant difference. However, after adjustment for each individual woman's rate of X aneuploidy in comparison to her paired uncultured result, no significant difference between the 72 and 96 hour rates of X chromosome aneuploidy was found. Differences between cell division in regard to culture time and cell cycle times of abnormal cells possibly varying from normal cells (Rooney and Czepulkowski, 1992) are not significant between 72

and 96 hour culture. This result gives preliminary indicators that the effect of culture on X chromosome aneuploidy for a given individual remains steady between the 72 and 96 hour culture period. This study suggests that most of the cultural effect on X chromosome aneuploidy occurs within the first 72 hours of culture.

As expected, the difference between the number of recalled pregnancies and the number of children each woman had borne was significant.
The spontaneous miscarriage rate of recognised pregnancies is often quoted at 15-20% (Edmonds *et al*., 1982) which our own data supports. However, Jorgensen and Klein (1988), claim that as many as 40% of positive pregnancy tests do not develop into a pregnancy. Because the human miscarriage (and aneuploidy) rates are related to maternal age, any demographic or social factors predisposing to child bearing later in life can act as indirect contributors to the rate of spontaneous miscarriage. Concurrently, a number of factors have been proposed in the demographic and sociological literature to explain the phenomenon of delayed child-bearing, including safe, effective contraception, changes in societal expectations of women in post-secondary education and the workforce, and an increased population of women 35 to 44 years of age (Frets, 2009). This study deals only with primary (biological) causes of aneuploidy, and so these secondary issues are not addressed by any data of this project.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUDING COMMENTS

The justification for this study was to provide a basis for interpreting X chromosome aneuploidy elucidated during cytogenetic analysis of lymphocytes from women of unknown fertility. X chromosome aneuploidy was determined in a cohort of 78 women of proven fertility using uncultured peripheral blood lymphocytes and lymphocytes cultured in the presence of the mitogen PHA for either 72 or 96 hours.

The levels of X chromosome aneuploidy were determined using DNA probes and FISH on peripheral blood lymphocytes. Aberrant findings within the laboratory were resolved by comparing results on paired uncultured and cultured cell samples. In this way discordant results were resolved without recourse to further specimen sampling with benefits for both the laboratory and the person being tested.

The main objectives of this study, as detailed in Chapter 1 (page 10), have been achieved. A significant statistical difference ($p<0.0001$) between the level of X chromosome aneuploidy in cultured and uncultured peripheral blood cells was observed. It was shown that a low background rate of X chromosome aneuploidy (<5%) was present in lymphocytes preparations from young women with proven fertility irrespective of whether the cells were cultured (with PHA mitogen) or not cultured. This is a significant finding, since the cut-off commonly used in routine diagnostic cytogenetic laboratories is < 10% X chromosome aneuploidy (Horsman *et al.*, 1987), which is suggested to be without reproductive significance in the phenotypically normal woman.The results from our study support the findings of Peschka et al. 1999 who propose the figure of < 6% X chromosome mosaicism should be considered normal.

Cognate analyses were also performed on the effects of age of the women tested, their gravidity and parity, and time of cell culture in the presence of PHA mitogen. These results are described in detail in Chapter 4. As expected the significant effects of culture and age on the level of X chromosome aneuploidy were clearly demonstrated with cultured samples from women $\geq$35 having a

significantly higher level of X chromosome aneuploidy that of their younger counterparts (≤35). The relative rate for X chromosome aneuploidy in cultured preparations was 2.33 with the highest overall rates occurring in the cultured preparations regardless of age. However, X chromosome aneuploidy detected in the uncultured cells was essentially unchanged with increasing age (up to 40 years).

These findings demonstrate that "cultural artefact" was significant even in a cohort of young fertile women, and that the blood culture method used in cytogenetic testing has a significant effect on X chromosome aneuploidy. It confirms previous work both on the impact of the culture process and that X chromosome loss occurs more frequently than gain (Nowinski *et al*., 1990; Richard *et al*., 1993; Guttenbach et al., 1995; Russell *et al*., 2007).

The age dependant component of X chromosome aneuploidy in the cultured cells detected in this study suggests a real interaction between some aspect of the cell culture environment, and some inherent quality of blood lymphocytes, specific to older women. As the inactive X (Lyon, 1961) is late replicating (Gilbert *et al*., 1962) and replication is only put to any functional test in this study when the cells are cultured *in vitro*. It may be hypothesised that some age-related reduction in the spindle's ability to attach to the inactive X in the abnormal environment of cell culture may be the cause of increased levels of X chromosome loss in culture. Any such effect would be absent from the uncultured cells. Such a spindle-based *in vitro* hypothesis would be analogous to that suggested by Battaglia *et al*. (1996), who suggest an *in vivo* meiotic generation of autosomal trisomy in older women.

This study compared the rates of X chromosome aneuploidy in uncultured peripheral blood lymphocytes and cells cultured in the presence of mitogen for 72 and 96 hours. After statistical adjustment for individual variation no significant difference was in found between levels of X chromosome aneuploidy in the 72 and 96 hour cultures. This data allows for a rational choice of culture duration that best accommodates the routine laboratory management schedule.

The cytogenetic methods used in this study, i.e. analysis of interphase cells from peripheral blood cells using chromosome specific FISH probes, permit the resolution of queries over X chromosome aneuploidy without resorting to the invasive technique of fibroblast culturing. FISH analysis permits the rapid

quantification of a large number of cells providing advantages in terms of producing accurate results relatively quickly. The use of FISH probes is already part of standard cytogenetic practice and it is therefore feasible to incorporate the method outlined in this project into routine laboratory practice, when required to explore the cause of low level X chromosome mosaicism (with an equivalent sample size of 500 nuclei to empower discrimination between 5% and 10% levels of aneuploidy). This is a finding of cytogenetic significance, since it eliminates the need to issue a 'qualified' abnormal report, request a second peripheral blood sample or alternate tissue sample (a biopsy).
Patient stress and clinical uncertainty will therefore be significantly reduced.

Many of these findings have been published in a peer reviewed paper entitled "Rate of X chromosome aneuploidy in young fertile women: Comparison of cultured and uncultured cell preparations using fluorescence *in situ*hybridisation" a copy of which is included in Appendix 1.

The strengths of this study include the use of a routine sample type (peripheral blood), in paired (cultured and uncultured) samples as well as the large number of cells examined (78,000 interphase nuclei in total). The carefully defined population of women with normal fertility also sets this project apart from other studies. The possibility of including a second locus specific DNA probe on the short (p) arm or the long (q) arm of chromosome X was considered but not pursued due to weaker fluorescent signals detected from locus specific probes compared with the centromeric probes. This avoided the possibility of confounding data from weak hybridisation that may have implied a possible structural X chromosome rearrangement.

Since this project was undertaken, Zotova *et al*. (2009) have used FISH on cultured and uncultured cells from women of reproductive age to examine aneuploidy for chromosomes 13, 18, 21 and X. They found a mean aneuploidy rate of 1.39%. They also confirm that the cultivating process influences the aneuploidy level. Homer *et al*. (2010) have suggested that the clinical threshold for incidentally diagnosed low level sex chromosome mosaicism is 6%. Mosaicism of ≈8% was shown to affect height and body mass index. They also found that although X chromosome mosaicism of < 30% did not impact on ovarian reserve, spontaneous fertility was still affected with a significant increase in first trimester pregnancy loss in women with 45,X/46,XX mosaicism.

More recently Homer et al. (2012) have found that 45,X/46,XX mosaicism between 6 – 28% does not have an adverse impact on IVF or ICSI among the women they studied who were referred for assisted reproductive technologies. They hypothesise that the lack of effect on the endocrine and exocrine functions of the ovary from X chromosome mosaicism in this range may be due to tissue limited mosaicism in the lymphocytes. They do however acknowledge that low level X chromosome mosaicism in this range is associated with premature ovarian failure and that only a comparison with women who conceived spontaneously would truly establish the absence of fertility impairment in women with 45,X/46,XX mosaicism.

The implications of the findings in this project are that similar or more pronounced effects of cell culture technology may be present in other patient groups and this needs further investigation. Further work clarifying the effects of culture on higher rates of X chromosome aneuploidy around points of clinical relevance such as women with various fertility problems and women with chromosomal abnormalities such as 47,XXXwould be useful. As fertility is an issue of increasing importance for older women (maternal age is increasing in Australia), further information on the effects of age and culture on X chromosome aneuploidy in women over 40 years of age is also needed.

Before reporting low level X chromosome aneuploidy obtained from standard culture,the impact of cultural artefact needs to be carefully considered. This is, especially so, in older infertile women where reported levels of X aneuploidy increased by cell culture effect may be misleading for clinicians or distressing for patients. It may be appropriate to confirm the true rate of X aneuploidy by direct analysis using FISH. Alternatively the relative rates of X chromosome aneuploidy found in the cultured cells of fertile female patients less than 40 years of age in this study could be used to define normative data of X chromosome aneuploidy.

In conclusion, in fertile young women this study found a low rate of X chromosome aneuploidy of < 5%, regardless of preparation technique, which is lower than the <10% previously reported. The effects of culture on the rate of X chromosome aneuploidy were also defined with cultural artefact producing more aneuploid X chromosome signals than age alone in women < 40 years. The < 10% cut-off commonly used for determining significance of low level aneuploidy may need to be reviewed considering this study's findings of < 5% aneuploidy in normally fertile women.

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APPENDIX 1

PUBLISHED PAPER

Rate ofX chromosome aneuploidy in young fertile women: Comparison of cultured and uncultured cell preparations using fluorescence*in situ* hybridisation

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Background: X chromosome aneuploidy <10% in female patients is a routinely used reporting limit in diagnosticcytogenetics. X aneuploidy (<10%) is commonly detected in women investigated for infertility or recurrent miscarriages.It is unclear if this aneuploidy is causally relevant or related to the culture process. Information about the background rateof X aneuploidy in young fertile women would be helpful in resolving this issue.

Aim: This study aimed to investigate the rate of X aneuploidy in young fertile women in cultured and unculturedsamples to determine if the commonly used <10% limit is relevant.

Method: Volunteers (aged 22–40 years) with proven fertility (n = 78) participated. The number of X chromosome signals in 500 cultured and 500 uncultured preparations were enumerated using FISH. Results: Significantly, all participants had <5% X aneuploidy in both preparations, X chromosome loss occurred (2.4%) more frequently than gain (0.7%). Cultured preparations had a mean of 2.1% cells with X chromosome aneuploidy (95% CI 1.9–2.3%) compared with a mean rate of 0.9% aneuploidy in uncultured preparations (95% CI 0.7–1.1%). The relative risk for cultured preparations having X aneuploidy compared with uncultured cells was 2.33 (P < 0.001) (95% CI 2.1–2.6).

Conclusion: Young fertile women had <5% X aneuploidy. The rate of X aneuploidy was higher in cultured (2.1%) compared with uncultured (0.9%) preparations (P < 0.001). This data may provide useful background information when considering low level X aneuploidy in other groups of women with clinical indications for karyotype.

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Original Article

Rate of X chromosome aneuploidy in young fertile women: Comparison of cultured and uncultured cell preparations using fluorescence *in situ* hybridisation

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Background: X chromosome aneuploidy <10% in female patients is a routinely used reporting limit in diagnostic cytogenetics. X aneuploidy (<10%) is commonly detected in women investigated for infertility or recurrent miscarriages. It is unclear if this aneuploidy is causally relevant or related to the culture process. Information about the background rate of X aneuploidy in young fertile women would be helpful in resolving this issue.
Aim: This study aimed to investigate the rate of X aneuploidy in young fertile women in cultured and uncultured samples to determine if the commonly used <10% limit is relevant.
Method: Volunteers (aged 22–40 years) with proven fertility (n = 78) participated. The number of X chromosome signals in 500 cultured and 500 uncultured preparations were enumerated using FISH.
Results: Significantly, all participants had <5% X aneuploidy in both preparations, X chromosome loss occurred (2.4%) more frequently than gain (0.7%). Cultured preparations had a mean of 2.1% cells with X chromosome aneuploidy (95% CI 1.9–2.3%) compared with a mean rate of 0.9% aneuploidy in uncultured preparations (95% CI 0.7–1.1%). The relative risk for cultured preparations having X aneuploidy compared with uncultured cells was 2.33 (P < 0.001) (95% CI 2.1–2.6).
Conclusion: Young fertile women had <5% X aneuploidy. The rate of X aneuploidy was higher in cultured (2.1%) compared with uncultured (0.9%) preparations (P < 0.001). This data may provide useful background information when considering low level X aneuploidy in other groups of women with clinical indications for karyotype.

Key words: aneuploidy, fluorescence *in situ* hybridisation, mosaicism, X chromosome.

Introduction

Clarifying the phenotypic relationships of chromosomal mosaicism is an important issue in cytogenetic diagnosis and genetic counselling.[1] True mosaicism is present when two or more chromosomally different cell lines occur within an individual.[2] However, assessing *in-vivo* patient status using *in-vitro* testing may be unreliable as the culture process may change proportions of abnormal cells[2] or the abnormal cell line may occur *ab initio in vitro*, resulting in 'cultural artefact'. Chromosome X loss may increase with longer culture time.[2] Chromosome X aneuploidy is also known to increase with age in men and women.[3–5]

A study (n = 104) by Horsman *et al.*[6] found low level X mosaicism occurred in 16% of infertile women. Larger studies have generally found lower rates of X chromosome mosaicism in women presenting for fertility treatments with reported rates of 4.5%,[7] 3.3%,[8] 3.2%[9] and 9.6%.[10] The data are insufficient to confirm suggestions that these findings may not have reproductive significance in phenotypically normal women.[11]

The rate of X chromosome aneuploidy in young, fertile women has neither been reliably determined[12] nor has the influence of the culture process on the level of aneuploidy been clarified. In a retrospective study by Nowinski *et al.*,[13] controls (n = 332) were examined for low level mosaicism; however, fertility status of these women was not clearly characterised. Russell *et al.*[5] (n = 655) only examined X chromosome loss and included women with fertility

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problems and Turner syndrome stigmata. Other lymphocyte culture studies have been limited by small sample size ($n = 21$)[14] or investigation of patients with abnormal phenotypes.[5,15]

Defining the baseline rate of low level X chromosome aneuploidy in fertile women is important as a reference point in considering findings in women investigated for clinical problems. This study aimed to provide information on the rate of X chromosome aneuploidy in young, fertile women by comparing cultured and uncultured preparations.

Methods

Ethics approvals were obtained from local institutions

Seventy-eight volunteers participated in the study. Eligible women were aged ≤40 years and had proven fertility. Blood samples were collected after obtaining informed consent. Proven fertility was defined as at least one natural pregnancy, no history of recurrent miscarriages (three or more consecutive miscarriages), or any fertility treatments (including ovulation tracking, clomiphene citrate, IVF, ICSI, or other invasive treatments) or referrals to a specialist infertility clinic. Women over 40 years were excluded in recognition of known age effects on sex chromosome loss.[4,5]

Cultured preparations were processed using standard cytogenetic procedures. Uncultured blood was treated directly with a hypotonic solution, 0.075 mol/L KCL for 10 min, before washing and fixing in methanol : acetic acid (3:1) using a method adapted from Jalal and Law.[16]

Cultured and uncultured cells were examined from each case using two-colour FISH analysis with centromere-specific probes for chromosome X and 9 according to manufacturer's instructions (CEP X and CEP 9 Vysis, Inc. Downers Grove, IL, USA).[17,18] The same probe batch and lot was used in all hybridisations.[19] Details of two-colour FISH analysis using a control probe on a chromosome other than the chromosome of interest have been previously described.[17,18]

Samples were examined as de-identified paired observations; 500 cells from both preparations were scored providing a total of 1000 cells per case. Only nuclei with two signals for the internal control were scored. Nuclei without two signals for the X chromosome were classed as aneuploid. These included cells with 1, 3, 4, or 5 X centromeric signals. This is consistent with Horiuchi *et al.*[17]

The number of cells counted was calculated using assumptions that the background rate of aneuploidy may not exceed 1% (±0.5%) with 99% confidence, similar to Tonecheva *et al.*[1] To attain a confidence level of 99%, it was estimated that counts from 421 cells are required. Analysis of 500 cells allows detection of a background rate of aneuploidy from as low as 0.4% to 1% in cells.

The sample size of 78 paired samples was chosen to allow detection of a rate ratio of 2.0 for X aneuploid cells associated with the 'cultured' cells with ≥80% power and 5% significance level using Poisson regression. This sample size was estimated assuming a minimum mean background rate of aneuploid cells between 0.4% and 1% (Power and Sample Size for Windows, PASS 2002, Kaysville Utah) and deemed appropriate considering the previously reported rates of X aneuploidy of 4.5%,[7] 3.3%,[8] 3.2%[9] and 9.6%.[10]

Continuous data were summarised using medians and percentiles or means and standard deviations. Categorical data were summarised using frequency distributions. Univariate comparisons between uncultured and cultured cells were conducted using the Mann–Whitney test for independent samples and the Wilcoxon Signed Ranks test for paired samples. Poisson regression analysis for paired samples was performed and covariate effects were summarised using rate ratios (RR) and their 95% confidence intervals (CI). SPSS (SPSS for Windows version 11.0, Chicago, IL, USA) and LogXact (LogXact version 5; Cytel, Cambridge, MA, USA) were used for data analysis. All *P*-values were two-sided and *P*-values <0.05 were considered statistically significant.

Results

A total of 78 000 interphase nuclei from the 78 participants were analysed using FISH for X aneuploidy. The median age of women participating in the study was 33 years (range 22–40) with 56/78 (71.8%) aged <35 years and 22/78 (28.2%) 35 years and older.

The hybridisation efficiency of the internal control was 98.8% in the uncultured nuclei and 99.4% in the cultured. No statistically significant difference in hybridisation rate between cultured and uncultured preparations ($P = 0.066$) was found. This falls within the range reported in a large multicentre study by Dewald *et al.*[20] who found an overall probe efficiency rate of 89.5–100% in interphase nuclei.

The frequencies of X chromosome signal loss or gain are shown in Table 1, with loss (2.36%) occurring more frequently than gain (0.66%). The rate of aneuploidy was significantly greater in cultured (2.12%) compared with uncultured cell (0.9%) preparations ($P < 0.001$).

The mean number of cells with aneuploid X chromosome signals in the uncultured group was 4.5 (95% CI 3.7–5.4), and in the cultured group was 10.6 (95% CI 9.5–11.7) (Table 2), a relative aneuploidy rate of 2.3 (95% CI 2.1–2.6) comparing cultured with uncultured preparations. The rate

Table 1 Frequencies of X chromosome aneuploidy from 'uncultured' and 'cultured' cell preparations in 78 women

	Number of X chromosome signals/cell					
	1	2	3	4	5	Total
Uncultured *n*	305	38 647	48	0	0	39 000
%	0.80	99.10	0.1	0.0	0.0	100%
Cultured *n*	604	38 175	212	8	1	39 000
%	1.56	97.88	0.54	0.02	0.0	100%

Table 2 Comparison of cells showing X chromosome aneuploidy in 'uncultured' and 'cultured' peripheral blood samples for all women and age < or ≥35 years

Mean aneuploidy/500 cells		Uncultured	Cultured	*P*-value
All *n* = 78	*n*	4.5 (IR, 2–6, R 0–19)	10.6 (IR 6.8–15, R 1–23)	<0.001
	%	0.91 (IR 0.4–1.2, R 0–3.8)	2.1 (IR 1.4–3.0, R 0–4.6)	<0.001
Age <35 years *n* = 56	*n*	3.8 (IR 1.3–5.0, R 0–17)	6.4 (IR 6.0–13.0,R 0–19)	<0.001
	%	0.76 (IR 0.3–1.0,R 0–3.4)	1.3 (IR 1.2–2.6,R 0–3.8)	<0.001
Age ≥35 years *n* = 22	*n*	9.9 (IR 3.8–8.3,R 2–19)	12.4 (IR 7.0–16.3,R 1–23)	0.001
	%	2.0 (IR 0.8–1.7,R 0.4–3.8)	2.5 (IR 1.4–3.3,R 0.2–4.6)	0.001

Medians, interquartile ranges (IR) and ranges (R) are shown.

of X chromosome aneuploidy was <5% for all women regardless of method of cell preparation. The range of aneuploid X chromosome signal patterns in uncultured samples was 0.0–3.8% and in the cultured samples was 0.2–4.6% and this difference was significant ($P < 0.001$).

The effect of maternal age and cell preparation (cultured or uncultured) on X chromosome aneuploidy was analysed. (Table 2). The lowest rate of aneuploidy was found in the younger women (<35 years) when uncultured cell preparations were used and a relative rate of 1.0 was assigned to this group (Table 3). All other combinations of cell preparation and maternal age showed a significant difference ($P < 0.001$) in X chromosome aneuploidy when compared with this rate. As shown in Table 3, uncultured preparations of cells from women aged 35 and older were associated with a relative rate of 1.67 (95% CI 1.35–2.07) for X chromosome aneuploidy. Culture had a more significant effect with cultured cells from women <35 having a relative rate of 2.59 (95% CI 2.21–3.03) for aneuploid X chromosome signals. The highest rate of aneuploidy occurred in cultured cells from women aged 35 years and over with a relative rate of 3.26 (95% CI 2.73–2.73-3.90).

Discussion

The strengths of this study were the use of a routine sample type, the large number of cells examined (78 000 interphase nuclei) and the defined population of women with normal fertility. This study found a low rate (<5%) of X chromosome aneuploidy in young fertile women independent of cell preparation method. The mean rate of X chromosome aneuploidy was 0.9% for uncultured samples and 2.1% for cultured samples. Significant effects of culture and age on the level of X chromosome aneuploidy were noted, with cultured samples from women ≥35 having the highest rate of aneuploidy. The relative rate for X chromosome aneuploidy in cultured preparations was 2.33 with the highest overall rates occurring in the cultured preparations regardless of age. These findings suggest that 'cultural artefact' was significant even in a cohort of young fertile women.

Table 3 Rate of X chromosome signal aneuploidy in women with combination of method and age

Method	Relative rate	95% CI	*P*-value
Direct preparation in women <35	1.00		
Direct preparation in women ≥35	1.67	1.35–2.07	<0.001
Cultured preparation in women <35	2.59	2.21–3.03	<0.001
Cultured preparation in women ≥35	3.26	2.73–3.90	<0.001

There are limited data reported on mosaic X chromosome aneuploidy in women with normal fertility. A small study ($n = 6$) of fertile women found a frequency of 0.12% for monosomy X and 0.05% for trisomy X[1]. A larger study ($n = 174$) found a frequency of 6.9% for monosomy X and 2.9% for trisomy X[13]. Morrel *et al.*[10] found X chromosome loss or gain in 22% of their normal control group. The fertility status was not reported for either of the groups used in the studies by Morel *et al.*[10] or Nowinski *et al.*[13] A commonly used cutoff in routine diagnostic cytogenetics, where a limit of 10% X chromosome aneuploidy is suggested to be without reproductive significance in the phenotypically normal woman, resulted from the work of Horsman *et al.*[6] and is referred to in Gardner and Sutherland.[11] In this study, an even lower rate of <5% aneuploidy was found in young fertile women from both cultured and uncultured cell preparations.

Previously reported effects of age on the karyotype[4,5,13] were confirmed in this study, with a higher rate of aneuploidy noted in women ≥35 years compared younger women. Further study of normally fertile women over the age of 40 may be helpful to assess the rate of aneuploidy in this group.

This study compared the rates of X chromosome aneuploidy in uncultured lymphocytes and lymphocytes obtained from standard culturing processes. It utilised the same sample type as used in routine chromosome analysis and employed a method commonly available in cytogenetics laboratories to further investigate aneuploidy. By using FISH a large number of cells can be rapidly counted, which facilitates further assessment of low level aneuploidy. Although lymphocyte analysis is standard cytogenetic practice, it should be noted that these findings can not address the issue of tissue limited mosaicism, and as such may not reflect chromosome constitution in the gonads.

Conclusions

A <10% cutoff is commonly used for determining significance of low level aneuploidy. This study found that in normal, fertile young women (≤40 years) the rate of X chromosome aneuploidy was even lower at <5%, regardless of preparation technique. The findings in this study suggest the need for further investigations to clarify the effects of chromosome culture on mosaic aneuploidy in other patient groups, including older women and those with infertility.

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APPENDIX 2

PATIENT INFORMATION SHEET

The Effect of Cell Culture on X Chromosome Mosaicism

Women who have had difficulty becoming pregnant or have had repeated miscarriages may have chromosome studies as part of their investigations. Abnormalities which can cause problems with pregnancy are occasionally found, but sometimes other variations are found which are harder to interpret as they might have no medical significance. One of the more common findings is a variation in the number of X chromosomes. Normally each woman has two X chromosomes in every cell, but sometimes a cell may lose or gain an X chromosome. Doctors often question if these small variations in X chromosome numbers are of any importance to the individuals fertility. They have never been shown to cause future medical problems. It is also uncertain if different laboratory methods actually cause some of this minor variation during culture of the cells.

We want to check the X chromosome numbers of women who have **not** had problems becoming pregnant to see if the same variations can happen in this normally fertile group. If the same level of variation were found in normally fertile women, this would help us to be able to reassure other women that these variations are nothing to worry about with regards to pregnancy.

There is limited information on what variation is normal in fertile women, but we expect that levels of up to 12% variation may occur. It is generally agreed that levels of 10% or less X chromosome variation do not mean anything significant.

We would like to check the X chromosome numbers in a number of women and will test different laboratory methods as well to see if they affect the result. This is a limited chromosome analysis, as we will simply count the number of X chromosomes and will not examine the structure of any chromosomes at all. This means we will not be doing a full chromosome analysis and will not be able to detect anything but variation in the numbers of the X chromosome.

We expect that this study will allow us to better define the variation in X chromosome numbers that are found in normally fertile women. This information will be useful for resolving the issue of X chromosome mosaicism if it arises during the routine performance of this test.

The Ethics Committees of Curtin University, King Edward Memorial Hospital & Princess Margaret Hospital, as well as the Mount Hospital, Mayne Health have all approved this study.

If you participate in this study, 7mls of blood will be collected from you. The results of the study will be published without identifying any participants by name. You will have the choice of receiving your individual result, which will state whether it was in the expected range or outside of this.

If "positive" finding outside the expected range of X chromosome variation greater than 12% is detected in your sample, we will inform you and offer genetic counselling to further explain this result. If levels of variation greater than 12% are detected, these are likely to reflect on the previously undetected level of X chromosome variation in normally fertile women.

It must be emphasised that, all study participants have had a history of normal fertility established and a even a "positive" finding of X chromosome variation greater than 12% should not impact on any current pregnancy or future fertility.

Once tested, your sample will be destroyed. All results will have names removed and no patient names will be used in publishing this work. If you wish to participate in this study please read and sign the attached consent form.

You may withdraw from this study at any time and your sample will be de-identified and incinerated.

Please contact
Kirralee Patton, Principal Investigator
Scientist in charge of Cytogenetics
Western Diagnostic Pathology
Ph: 9317 0921

Thank you for your assistance

APPENDIX 3

GENETIC MATERIAL PATIENT INFORMATION SHEET

Genetic material (DNA) determines an individual's characteristics. It is present in almost every cell of the body. Cytogenetic scientists visualise and analyse DNA as larger units called chromosomes.

Chromosomal material will be extracted from your blood sample and stored at 4°C for the duration of the study (approximately one year).

This genetic material will **only**be used in the study - **Variation in X chromosome numbers in normally fertile women and the effect of different laboratory methods on these results.**

After completion of the study your sample will be de-identified and disposed of by incineration.

You will be asked if you would like to receive your individual results from this study or not. If your result falls outside the range of variation predicted in fertile women you will be invited to contact the Department of Genetic Services to discuss your results further.

This study has no potential to detect non-maternity or non-paternity.
No information about family members is required.
Health information gathered will not be passed onto to family members with your consent.

You may withdraw from this study at any time and your genetic material will be de-identified and incinerated. Thank you for your assistance.

Please contact

Kirralee Patton, Chief Investigator of the study
Scientist in charge of Cytogenetics
Western Diagnostic PathologyPh: 9317 0921

APPENDIX 4

PATIENT CONSENT FORM

The Effect of Cell Culture on X Chromosome Mosaicism

I consent to the collection of my blood, from which genetic information will be extracted and stored for chromosome studies.

I consent for my chromosomes being used for research for this study.

I have read and understood the Information Sheet given to me. Any questions I have asked have been answered to my satisfaction.

I understand that once tested, my blood sample will be destroyed and any results obtained will be de-identified.

I agree that research data gathered from the results of this study may be published, provided that names are not used.

I understand that the numbers of X chromosome's present in cells can vary and that this study will only count the numbers of X chromosomes present in my cells. This study will not examine my chromosomes in detail and I understand it is not a full chromosome analysis.

I ……,agree to participate in this study.

[ ] I would like to receive my own result

[ ] I do not wish to receive my own result

Signed …….. Date……..

I have explained the above to the signatory who stated that she understood the same.

Witness ……..Date…….

APPENDIX 5

DNA CONSENT FORM FOR GENE STUDIES

I consent to the collection of my blood, from which genetic information will be extracted and stored for chromosome studies.

I consent for my chromosomes being used for research into

Variation in X chromosome numbers in normally fertile women and the effect of different laboratory methods on these results.

Name of Subject……

Signature…… Date ……..

Witness…..…… Date ……..

APPENDIX 6

DATA COLLECTION ON STUDY SUBJECTS

Re: Variation in X chromosome numbers in normally fertile women and the effect of different laboratory methods on these results.

Name: **……**

Address: …….

Telephone number: …….

Date of birth: …….

In order for us to classify the fertility of study participants, we would appreciate it if you would answer the following questions regarding your obstetric history. All responses are kept strictly confidential. We thank you for your assistance in this research.

How many times have you been pregnant?…….
(Please include current pregnancy and all previous pregnancies that have not gone to full term)

How many children do you have?……..

Have you ever used fertility treatments?…… (For example, Ovulation Tracking, Clomiphene, IVF, and ICSI)

Have you had 3 or more consecutive miscarriages?
……

APPENDIX 7

THE HYBRIDISATION EFFICIENCY OF THE CHROMOSOME 9 CONTROL PROBE IN NUCLEI FROM UNCULTURED CELLS

IDNUMBER	1 x 9 signal	2 x 9 signal	3 x 9 signal	% 1 x 9 signal	% 2 x 9 signal	% 3 x 9 signal	#Aberrant 9 signal	%Aberrant 9 signal
87D	8	492	0	1.6	98.4	0	8	1.6
88D	0	499	1	0	99.8	0.2	1	0.2
89D	6	493	1	1.2	98.6	0.2	7	1.4
90D	3	497	0	0.6	99.4	0	3	0.6
92D	5	495	0	1	99	0	5	1
93D	4	496	0	0.8	99.2	0	4	0.8
94D	8	491	1	1.6	98.2	0.2	9	1.8
95D	2	498	0	0.4	99.6	0	2	0.4
96D	9	491	0	1.8	98.2	0	9	1.8
97D	12	488	0	2.4	97.6	0	12	2.4
AVERAGE	**5.7**	**494**	**0.3**	**1.14**	**98.8**	**0.06**	**6**	**1.2**
STD DEV	**3.62**	**3.56**	**0.48**	**0.72**	**0.71**	**0.09**	**3.56**	**0.71**
95% CI	**2.25**	**2.21**	**0.29**	**0.45**	**0.44**	**0.06**	**2.20**	**0.44**

APPENDIX 8

HYBRIDISATION EFFICIENCY OF THE CONTROL CHROMOSOME 9 CEP IN THE CULTURED NUCLEI

ID NUMBER	# 1 x 9 signal	# 2 x 9 signal	# 3 x 9 signal	# 4 x 9 signal	% 1 x 9 signal	% 2 x 9 signal	% 3 x 9 signal	% 4 x 9 signal	#Aberrant 9 signal	%Aberrant 9 signal
87C	3	497	0	0	0.6	99.4	0	0	3	0.6
87C	1	495	4	0	0.2	99	0.8	0	5	1
89C	8	492	0	0	1.6	98.4	0	0	8	1.6
90C	1	498	1	0	0.2	99.6	0.2	0	2	0.4
92C	1	499	0	0	0.2	99.8	0	0	1	0.2
93C	4	496	0	0	0.8	99.2	0	0	4	0.8
94C	1	499	0	0	0.2	99.8	0	0	1	0.2
95C	1	499	0	0	0.2	99.8	0	0	1	0.2
96C	1	498	0	1	0.2	99.6	0	0.2	2	0.4
97C	4	495	0	1	0.8	99	0	0.2	5	1
AVERAGE	**2.5**	**496.8**	**0.5**	**0.2**	**0.5**	**99.36**	**0.1**	**0.04**	**3.2**	**0.64**
STD DEV	**2.32**	**2.29**	**1.27**	**0.429**	**0.469**	**0.46**	**0.25**	**0.08**	**2.29**	**0.46**
95% CI	**1.44**	**1.43**	**0.79**	**0.269**	**0.289**	**0.29**	**0.16**	**0.052**	**1.429**	**0.29**

APPENDIX 9

CHROMOSOME X ANUEPLOIDY RAW DATA IN UNCULTURED CELLS INCLUDING AGE, GRAVIDITY AND PARITY

ID Number	Age	X	XX	XXX	Total	Gravida	Para
14D	25	15	483	2	500	5	5
15D	39	9	489	2	500	3	3
16D	36	18	481	1	500	3	3
17D	39	10	489	1	500	3	1
18D	29	9	491		500	1	1
19D	28	9	489	2	500	2	2
20D	31	5	494	1	500	6	3
23D	38	3	496	1	500	5	3
24D	35	15	485		500	5	4
26D	37	5	491	4	500	3	1
27D	35	4	492	4	500	2	1
28D	32	4	495	1	500	1	1
29D	37	5	494	1	500	1	0
30D	28	8	489	3	500	1	1
31D	37	2	495	3	500	1	1
32D	23	5	495		500	1	?
33D	26	6	494		500	2	1
34D	38	8	492		500	2	1
35D	35	6	493	1	500	2	1
36D	31	4	494	2	500	3	1
37D	32	5	495		500	3	2
38D	34	1	498	1	500	2	1
39D	36	3	495	2	500	2	1
40D	35	4	496		500	1	0
41D	31	2	497	1	500	1	0
42D	22	5	494	1	500	8	3
43D	30	5	495		500	1	1
44D	23	7	493		500	2	1
45D	32	8	492		500	3	1
46D	35	1	499		500	3	2
48D	32	4	496		500	2	1
49D	33	7	493		500	5	2
50D	35	5	495		500	2	1
51D	32	4	496		500	2	0
52D	32	4	496		500	2	1
54D	32	2	498		500	2	1
55D	34	4	496		500	4	2
56D	30	4	496		500	3	2
57D	32	3	497		500	3	1
58D	34	2	498		500	2	1

ID Number	Age	X	XX	XXX	Total	Gravida	Para
59D	30	2	498		500	2	1
60D	33	1	499		500	2	1
61D	33	1	499		500	3	2
62D	31	5	495		500	3	1
63D	31	1	498	1	500	2	1
64D	33	2	498		500	3	1
65D	30	1	499		500	2	1
66D	34	4	495	1	500	2	1
67D	35	1	499		500	4	3
68D	33		500		500	4	3
69D	29	4	496		500	5	1
70D	33	3	497		500	3	2
71D	32	2	498		500	2	1
72D	30	2	498		500	4	1
73D	33	1	499		500	3	1
74D	24	2	498		500	3	1
75D	31	1	499		500	3	1
76D	31	2	498		500	3	1
78D	40	2	495	3	500	2	1
80D	33	1	499		500	4	2
81D	32	1	498	1	500	2	1
82D	33	1	499		500	2	1
83D	32	3	497		500	2	0
84D	32	2	494	4	500	3	2
85D	36	1	497	2	500	2	2
86D	36		500		500	2	1
87D	37	1	498	1	500	1	2
88D	34		500		500	4	1
89D	33		499	1	500	2	1
90D	34	5	495		500	1	1
91D	34	2	498		500	4	3
92D	23	6	494		500	1	1
93D	33	1	499		500	1	1
94D	32	1	499		500	3	3
95D	32	1	499		500	1	1
Sums		**322**	**4975**	**50**			
%		**6.5%**		**1.0%**			**7.5%**
Total		**305**	**38647**	**48**	**39000**		
%		**0.80%**	**99.10%**	**0.10%**			

APPENDIX 10

CHROMOSOME X ANUEPLOIDY RAW DATA IN CULTURED CELLS INCLUDING CULTURE TIME

ID Number	X	XX	XXX	XXXX	XXXXX	Total	1= 72hr 2=96hr
14C	11	487	2			500	1
15C	9	488	3			500	1
16C	7	485	7	1		500	1
17C	15	483	2			500	1
18C	12	487	1			500	1
19C	5	494	1			500	1
20C	10	485	5			500	1
23C	10	485	5			500	1
24C	8	486	4	2		500	1
26C	17	480	3			500	1
27C	9	482	9			500	1
28C	9	487	4			500	1
29C	6	493	1			500	1
30C	6	492	2			500	1
31C	14	484	2			500	2
32C	6	493	1			500	2
33C	10	490				500	2
34C	10	484	6			500	1
35C	16	477	7			500	1
36C	8	491	1			500	1
37C	5	489	6			500	1
38C	6	488	6			500	1
39C	11	489				500	1
40C	7	493				500	1
41C	15	483	2			500	1
42C	8	491	1			500	1
43C	5	490	5			500	1
44C	2	498				500	1
45C	3	494	3			500	2
46C	4	494	2			500	1
47C	5	492	3			500	1
48C	9	491				500	1
49C	14	482	4			500	1
50C	8	492				500	1
51C	9	485	6			500	1
52C	5	494	1			500	1
53C	13	486	1			500	1
54C	3	494	3			500	1
55C	8	489	3			500	1
56C	6	492	2			500	1
57C	8	488	4			500	2
58C	15	482	3			500	1
59C	5	491	4			500	1

ID Number	X	XX	XXX	XXXX	XXXXX	Total	1= 72hr 2=96hr
60C	14	481	5			500	2
61C	12	485	3			500	2
62C	7	492	1			500	2
63C	15	484	1			500	2
64C	5	492	3			500	2
65C	16	482	1	1		500	1
66C	16	482	2			500	2
67C	13	481	5	1		500	2
68C	8	492				500	1
69C	6	494				500	1
70C	10	489	1			500	1
71C	12	486	2			500	1
72C	5	495				500	1
73C	4	494	2			500	2
74C	3	495	2			500	2
75C	7	493				500	2
76C	3	494	3			500	1
77C	8	489	3			500	1
78C	5	493	2			500	1
80C	3	495	1	1		500	2
81C	6	491	3			500	1
82C	2	498				500	1
83C	1	496	3			500	2
84C	1	494	5			500	2
85C		495	5			500	2
86C	1	499				500	1
87C	11	486	3			500	1
88C	5	488	7			500	1
89C	7	484	9			500	1
90C	7	491	2			500	2
91C	2	493	4	1		500	2
92C	3	497				500	2
93C	8	488	2	1	1	500	2
94C	2	496	2			500	2
95C	4	491	5			500	2
Sums	**50**	**4913**	**34**	**2**	**1**		**16**
%	**1.0%**		**0.7%**	**0.0%**	**0.0%**		
Total	**604**	**38175**	**212**	**8**	**1**	**39000**	
%	**1.56%**	**97.88%**	**0.54%**	**0.02%**	**0.00%**		