DIFERENTES FRECUENCIAS DE EXPRESION DE RUPTURAS CROMOSOMICAS Y SITIOS FRAGILES EN DOS DIFERENTES MUESTRAS, SALUDABLE E INFERTIL

DIFFERENT FREQUENCIES OF FRAGILE SITES AND CHROMOSOME BREAKAGE EXPRESIÓN IN TWO DIFFRENT SAMPLES HEALTHY AND INFERTILE

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Proyecto de Grado

GENIS ANDRES CASTILLO VILLAMIZAR

Dirección

CLARA INES VARGAS CASTELLANOS, Msc.

Codirección

OLGA MARIA MORENO, Biol.

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Genis Andres Castillo Villamizar[**]

Keys words: Fragile sites, 3p14, Healthy, infertile.

RESUME:

The expression of fragile sites and others chromosomal breaks were evaluated in peripheral lymphocytes of one hundred selected healthy individuals and compared with fragile sites expression and chromosomal breakage of sixty-two infertile individuals, using conventional cytognetic techniques. An average of 20.25 and 40.44 breaks per individual were registered for healthy and infertile sample respectively, showing a significant difference in the expression rate of chromosomal breakage of the samples. Neither age nor sex influenced the expression of the chromosomal breakage in the analyzed groups. We identified 298 bands implied with breaks, forty five were statistically significant fragile sites, 43 were identified as common fragile sites and two as rare-fragile sites, the most common fragile site was the named 3p14 founded in all individuals of the infertile sample and in the 98% of the healthy individuals, fragile sites 6q26, 16q23 and 7q32 had a high frequency in the pooled data. Theses results are in accordance with other findings for Turkish and Chinese population. The major part of healthy individuals shown values of chromosome breakage from 15 to 20 breaks per 100 analyzed cells establishing, a parameter for the people in a healthy condition, giving us a new tool for futures comparisons in people suffering any kind of chromosomal damage or related phenomena in our region.

[**] Facultad De Ciencias, Escuela De Biología. Clara Inés Vargas Castellanos. Olga Maria Moreno.

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Genis Andrés Castillo Villamizar[**]

Palabras clave: Sitios frágiles, 3p14, saludable, infértil.

RESUMEN

La expresión de sitios frágiles y otras rupturas cromosomales fueron evaluadas en linfocitos de sangre periférica de 100 individuos saludables seleccionados y comparada con las frecuencias de expresión de 62 individuos infantiles, usando las técnicas citogenéticas convencionales. Se encontraron diferencias significativas en el promedio d rupturas y sitios frágiles entre las muestras analizadas un promedio de 20.25 y 40. 44 rupturas por individuo en las muestras saludables e infértil respectivamente. Se encontró que ni la edad ni el sexo influenciaron la tasa de expresión de sitios frágiles de los individuos analizados. Identificamos 298 bandas implicadas con rupturas de las cuales 45 fueron sitios frágiles estadísticamente significativos, de estos 43 pertenecen a los sitios frágiles comunes dos a los Sitios frágiles raros. El sitio frágil más común fue 3p14 que se presento en todos los individuos de la muestra infértil y en el 98% de los individuos saludables. Otros sitios frágiles 6q26, 16q23 y7q32 también presentaron una alta frecuencia en los datos registrados. Nuestros resultados son muy similares a los hallados por otros autores para las poblaciones turca y china. La mayor parte de los individuos saludables mostraron valores de rupturas entre 15 y 20 en 100 células analizadas, estableciendo un parámetro para la condición saludable, dándonos una herramienta una nueva herramienta para futuras comparaciones en personas que sufren algún tipo de daño cromosómico o fenómenos relacionados de nuestra región.

[**] Facultad De Ciencias, Escuela De Biología. Clara Inés Vargas Castellanos. Olga Maria Moreno.

INTRODUCTION

Many studies have shown that spontaneous chromosome gaps and breaks are not distributed at random along the chromosomes (Mattei et al., 1979) but they seem to be located at specific positions known as fragile sites (FS). FS are loci which are observed as nonrandom discontinuities of chromatin in metaphase chromosomes. These discontinuities can appear spontaneously or under appropriate culture conditions for example by exposing the cells to chemical agents (Sbrana and Musio, 1995; Porfirio et al., 1991; Sutherland and Hecht, 1985; Aula and Von Koskull, 1976). FS may be classified according to their prevalence and their mode of *in vitro* induction. According with their prevalence, they may be classified as rare (r-fs) or common fragile sites (c-fs). The r-fs are observed in the population with a frequency between one to five percent while c-fs may be observed in all individuals, be a component of normal chromosome structure, showing a high inter-individual variation (Denison et al., 2003; Sutherland et al., 1998, Sutherland and Richards, 1995; Hecht, 1991, 1986). According with their mode of *in vitro* induction, FS can be grouped on the basis of the various culture conditions that can be used for their expression into c-fs-like aphidicolin inducible (APC), bromodeoxyuridine (BrdU) inducible, 5-azacytidine inducible and r-fs distamycin A inducible, adenovirus type 12 inducible and folate sensitive (fs-FS). The latter can be expressed under low concentrations of folate, low or high concentrations of

thymidine or under other chemical agents such as methrotexate (MTX) and fluorodeoxiuridine (FudR) (Sutherland and Richards, 1999; Kubo et al., 1998; Tomas et al., 1984; Pranati et al., 1993; Sutherland and Hecht, 1985; Hecht and Glover, 1984; Barbi et al., 1984).

Since the discovery of chromosome breakage distribution and fragile sites (Mattei et al., 1979), many investigations have addressed their biologic and clinical significance. For example, it has been shown that FS are highly conserved among several mammal species such as felines, domestic pigs (*Sus scrofa)* and primates (*Pan troglodytes*, *Gorilla gorilla*, *Pongo pigmeus*) (Kubo et al., 1998; Dutrillaux, 1997; Yang and Long, 1993; Smeets and Van de Klundert, 1990; Miro, 1987) and it has been suggested that FS may play a role in the chromosomic reorganization in these species. In human beings, the clinical and biological significance have been studied. It has been observed that some FS may be implicated in genomic instability and some of them have been associated with chromosomic changes and cancer. For example, a limited number of FS have been commonly associated with several forms of cancer such as lymphoblastic leukemia, Hodking's lymphoma, Burkit's lymphoma, ovaric carcinoma and neuroblastom, among others (Fundia and Larripa, 1996; Jones et al., 1995; Chary-Redy et al., 1994; Yunis and Soreng, 1984). Other FS

seem to be implicated with other kind of diseases (Sutherland and Baker, 2000; Sutherland and Richards, 1999; Jones et al., 1995; Smeets and Van de Klundert, 1990; Yunis and Soreng, 1984). For example, the fragile site Xq 27.3 has been implicated in the fragile X syndrome, Xq 28 has been implicated with a moderate form of mental retardation and FS 11q 23.3 has been associated with the Jacobsen syndrome (Arlt et al., 2003; Agulhon et al., 1999; Hansen et al., 1997; Hirst et al., 1994). Other FS may be related with behavioral diseases such as autism and schizophrenia (Arrieta et al., 2002; Hsiang et al; 1998; Chen et al., 1998; Arrieta et al., 1996), even a link between a high chromosomic

fragility, spontaneous abortions and Fanconi anemia has been reported, showing that the high FS expression keeps a relationship with the infertility in couples with spontaneous abortions. Lastly some authors have established a relation between the genetic damage in molecular and cytogenetic level and the infertility; however the complete genetic action mechanism that is acting in the whole process is not clear, and more investigation in this field is necessary. (Joffe, 2003; Schmid et al., 2003; Anderson et al., 1997; Toncheva et al., 1991; Schlegelberger et al., 1989).

In spite of all these studies of association of FS, chromosomal damage and disease, our knowledge in this matter is still limited. For this reason, the discovery of a fragile site in a patient often causes anxiety and uncertainly on whether or not the patient will develop any form of cancer or any other disease, even infertility. It has been therefore suggested that the association among FS and disease should be fully investigated (Sutherland and Baker, 2000).

Another aspect of FS that has been the focus of recent research is the molecular basis of the chromosome breakage. So far, seven r-fs and six c-fs have been characterized at the molecular level. Two of the seven r-FS (FRA16B and FRA10B) are non-folate sensitive and induced by distamycin A and/or bromodeoxyuridine (BrdU). When these two r-FS were cloned and characterized, it was observed that they consisted of polymorphic AT-rich minisatellite repeats and their expression seemed to be associated with the expansion of one or more of these repeats up to several kilobases (Zlotorynski

et al., 2003; Sutherland and Richards 1999; Hewet et al., 1998). The other five r-FS were found to consist of expanded tandem CGG microsatellites repeats (<200 copies) (Sutherland, 2003; Sutherland and Richards, 1999; Nancarrow et al., 1995). These repeats are capable of adopting unusual structures, perturbing the DNA elongation and replication *in vivo* and *in vitro* (Zlotorynski, et al.,

2003); the six c-FS have been cloned and characterized and their cytogenetic expression (gaps and constrictions) have been found to span hundreds to thousands of kilobases. Recently, some authors have shown that the high DNA helix flexibility and the molecular expansions of theses CGG and AT repeats contribute to the chromosomic fragility and so perturb the replication and reparation mechanism of the DNA (Zlotorynski, et al., 2003; Sutherland and Richards 1999; Hewet et al., 1998). The cytogenetic and molecular characterization of FS is still limited and there are a number of interesting phenomena not only related to FS but to similar lesions on chromosomes that deserve further investigation (Sutherland and Baker, 2003).

In this sense, this work seeks to determine a possible contribution of FS and other forms of chromosomic breakages to infertility . We show that there is a statistically significant difference in the expression of FS and chromosome breakage between two samples fertile and infertile identifying those FS with higher frequency. This study gives a basis for further research on the possible association among the FS and infertility.

In the same manner this work gives an approximation to the normal frequencies of the chromosomal damage in a healthy sample, since the

frequency distribution of FS vary from one population to another it is necessary to know the frequency and variability of the FS and chromosome breakage in normal population for a future evaluation of the FS high expression frequency and its clinical significance in cancer or another abnormality in the same population (Güven et al., 1999).

MATERIALS AND METHODS

For the control group, a sample of one hundred healthy volunteers (50 males and 50 females) from the Colombian department of Santander was studied (previous personal authorization). The relatively small sample used in this research, as in other previous studies, is due to logistic and monetary problems that make the analysis of large samples impractical. Each individual was selected on the basis of inclusion and exclusion criteria that could affect the frequencies of chromosomic fragility (Hecht, 1991; Sbrana, and Musio, 1995; Tedeschi et al., 1993). The individuals' age ranged from 18 to 45 years and these were subdivided into two age groups (18-30 and 31-45) to carry out a correlation test between age and chromosomal damage. The individuals were not under any form of medication or genotoxic exposition, were not smokers nor coffee or tea drinkers. They were in a healthy condition and did not

experience any form of disease for at least 15 days before the sample was taken. In addition, the women within this group were not under any form of hormonal contraceptive. For the other study group, 62 infertile persons were selected. Only those persons whose infertility was likely to be caused by genetic damage and not by any other medical condition were included in this group. Toncheva (1991) used for his comparisons a control group of 15 subjects with offspring, we did not used necessary people with offspring because as it was mentioned above, chromosome breakage average depends of many variable factors and analyzing only people with offspring we would not know what was the frequency of chromosomal damage when they got their offspring because the frequency can change in time and space and is affected for the environmental factors (Sbrana and Musio,1995; Hecht, 1991;Yunis and Soreng, 1987, Marlhens et al., 1986). In the same way, it is not convenient to use pregnant women for the analysis because the hormonal level plays an important role in the expression of the common fragile sites (Takashi et al, 1991).

Peripheral blood lymphocytes from all individuals were incubated at 37°C in RPMI –1640 medium supplemented with bovine fetal serum and phytohemaglutinin. 72h later, methtrotexate MTX was added at a final concentration in the culture medium of 1 μM. As an internal control for the MTX activity, 15% of the samples were randomly selected and processed

without the presence of MTX in the culture medium. Standard cytogenetic techniques were used for harvesting, slide preparation and G banding following a modification of the protocol described by Rooney and Czepulkoeski (1987). One hundred G banded complete metaphase spreads at the 350-400 band level were scored for gaps and breaks on chromosomes for all samples. Gaps and breaks were recorded and localized according to the International System for human Genetics nomenclature (ISCN, 1995). FS identification was made using the complete fragile sites list in the Genome Data Base GDB (Arrieta et al., 2002; Denison et al., 2003)

Although there are different methods for the statistical identification of FS (Tai and Wang, 1998; Bohm et al., 1995; Jordan et al., 1990; Fundia and Larripa, 1989), we used a method based on the Poisson distribution (Mariani, 1989) to estimate, in both sample sets, the minimal number of events that must be present at a given band to be considered as a significant FS,. We then used a Z test to compare the average frequency of FS between healthy and infertile samples. Additionally, a correlation test was used between the age and the chromosomal damage.

RESULTS

Overall frequencies of chromosomal gaps and breaks

The numbers of chromosome breaks and gaps were very different between the healthy sample (HS) and the infertile sample (IS). 2025 breaks and gaps were recorded on 10000 analyzed metaphases for HS and 2501 breaks or gaps on 6200 analyzed metaphases for IS. On average, 0.205 and 0.211 breaks per cell were registered for females and males respectively in HS, while for the infertile sample an average of 0.393 and 0.404 breaks or gaps per cell for males and females respectively were observed. No significant differences for the rate of breaks or gaps between males and females within each sample set were found. Table1 shows the mean frequency of chromosomal gaps and breaks per cell and per individual for each sample set.

Statistically significant differences between the mean frequency of chromosomal breaks and gaps between individuals were found ($Z_{61, 99} = -11.32$, $p < 0.05$), showing a high chromosome breakage level for IS compared with HS. The chromosomal damage ranged from 0.04 to 0.4 breaks per cell in HS. On the other hand, IS had breakage values per cell ranging from 0.13 to 0.85.

The distribution of the chromosomal breaks or gaps within each sample set is shown in figure 1. The majority of the HS individuals showed breaks and gaps ranging from 15 to 20 breaks while the IS showed a range from 30 to 40 breaks per individual in the most cases.

The internal control showed a difference in more than 80% in chromosome breaks and gaps compared with the HS and IS. It did not found any statistical correlation between the age and the number of chromosome breaks or gaps ($p > 0.05$; $r = 0.02$).

Chromosomal location and frequencies between HS and IS

According to the method based on the Poisson distribution (Mariani, 1989), the minimal number of events needed for a band to be considered a significant expressed FS was five for the HS and six for IS. We found 285 bands implicated in breaks or gaps for HS and 298 in IS, the location of 89 of these bands overlapped with FS previously reported in GDB. Table 2 shows the chromosomal locations and the number of the 45 significant chromosomal gaps or breaks expressed as FS in at least one sample set. Of these 45 statistically significant FS identified, 43 corresponded to c-FS and 2 to r-FS. Most FS presented a higher expression in the IS than in the HS. In the HS, the 30.5 % of the chromosomal lesions were localized at 3p14 (FRA 3B). Similarly, 32.35 %

of the chromosomal breaks or gaps observed in the IS were located at the same position. The FS localized at 6q26 (FRA 6) was found in the 52% and 58% of the persons in the HS and IS respectively. Other fragile sites such as 7q32 and 16q23 showed a high number of breakage in both sample sets.

DISCUSSION

In the present study we have shown that there is an increase in the mean frequency of chromosomal breakage in the IS compared with the HS. Toncheva (1991) also found an increase in FS expression in couples with spontaneous abortions. Recently some authors have noted that an increase in the different forms of genetic damage, including DNA breaks and chromatin fragmentation, produced by disturbances in the DNA strand, may be a molecular mechanism for the FS expression (Zlotarinski et al., 2003; Arlt et al., 2003). This mechanism could be also considered as a cause of infertility in people who can not get offspring (Schimd et al., 2003). An important aspect of the infertility caused by genetic damage is the relationship between infertility and the pollutants since they having the potential to produce DNA damage in different kinds of cellular lines including germinal and somatic cells (Joffe, 2003). The expressions of the chromosome breakage, including FS expression, are a good quantitative measure of the genotoxicity and so allow the evaluation of the genotoxic effects of some pollutants in a population (Sbrana and Musio, 1995).

The genetic instability represented here as chromosome breakage depends on multiple factors such as ethnic origin, environmental conditions and genetics factors (Smeets and Arets, 1990; Sutherland and Hecht, 1985; Hsu, 1983). This shows the importance of evaluating the level of genetic damage in a sample of healthy people in order to know whether or not there is a risk factor associated with genetic stability in the same population using as parameter the values recognized for HS. Many works have studied the effect of some drugs, pesticides and chemical agents as increasing factors of chromosomal damage. This kind of environmental agents are virtually in everyplace thus making the study of the normal level of chromosomal damage in a population a priority (Güven et al., 1999; Sbrana and Mussio, 1995; Tedeschi et al., 1993). So far, a few papers have studied the frequency of chromosomal breaks in samples of healthy people from a population and have found similar expression rates of FS as in the present study with a few differences. For example, in each healthy population studied, 3p14 was reported as the most frequent broken band representing 17.8% of the ruptures in healthy Turkish individuals and 30.5% in healthy Colombian individuals (Güven et al., 1999; Takahashi et al., 1990). Our results show that the frequency of chromosomal breaks and gaps is higher in IS than HS. We can not rule out the possible existence of an external factor affecting the reproductive capacity of the infertile sample. Anderson et al.

(1997) showed the harmful effect of some toxins on human lymphocytes and sperm that have as final effect a reduction in the reproductive capacity.

Also in this study, the expression of chromosome breakage between males and females was compared. Although other authors have observed a sex influence on FS expression (Güven et al., 1999; Chary-Redy et al., 1994), we did not find any difference in the expression frequencies of general chromosome damage or specific FS between sexes. Smeets and Merkx (1990) who worked with 82 normal healthy individuals did not find differences between males and females, nevertheless Smeets and Arets (1990) showed that XX cell lines had bigger expression of FS than XY cells by studying common fragile sites in cultured peripheral lymphocytes of a true whole-body human chimera. This result suggests that the genetic factors may play an important role in the expression of common fragile sites.

Many investigators have studied the effect of age on the frequency of chromosomal aberration *in vitro* and some of them have found that the FS greatly increase with the age (Güven et al., 1999., Marlhens et al., 1986). However, others have shown that the age is not influencing the expression of FS (Smeets and Merkx, 1990). In this research we investigated the effect of age by comparing the number of chromosome breaks and gaps of two age groups.

Our results suggest that there is not a statistically significant correlation between the number of gaps and breaks and age. It would be worthy to review of the discrepancies between the different studies, including ours may be due to different experimental designs and sample sizes that do not allow the comparison of data or extract general conclusions.

In relation with the expression and distribution of FS, 3p14 was the most frequent site in both samples sets since it was present in all IS individuals and in the 98.3% of the HS. Other fragile sites such as 6q26, 16q23 and 7q31 showed a higher frequency compared with the other statistically significant FS in HS and IS. Except for the high frequency of 6q26, our results are in concordance with Güven et al. (1999), since 43 out of the 45 statistically recognized FS in our study were reported as FS in GDB. The bands 6q25 and 9q34 are not in the GDB but are reported as fragile sites in other previous works (Simonic and Gericke, 1996). The 3p14 site was found as the most common and frequent in our analysis as well as in other studies (Arlt et al., 2003; Denison et al., 2003; Güven et al., 1999; Hsiang et al., 1998; Simonic and Gericke 1996; Sbrana and Musio, 1995). It has been suggested that this site may represent a FS cluster in a small chromosomal region whose microscopic observation requires a higher resolution not provided by the conventional cytogenetic techniques (Ohta et al., 1996). The clinical significance of this site

is its potential involvement in several malignances, including some cancers, exhibiting the importance of the constant vigilance on the rates of chromosome breakage in our populations (Güven et al.1999; Sundareshan and Augustus, 1996; Fundia and Larripa, 1996). Based in the behavior of common fragile sites *in vitro* and their localization, many authors have suggested that the FS play a mechanistic role in the chromosome rearrangements involved in cancer (Fundia and Larripa, 1996; Yunis and Soreng, 1984); Artl et al. (2003) and Sutherland (2003) reviewed important aspects about fragile sites and highlighted the importance of the study of this phenomenon.

Finally, although there is a difference in the chromosome breakage and expression frequencies of FS between the healthy and the infertile sample sets used in this study that may be important in some aspect of the human reproductive health, it will be necessary to carry out further research to get a better understanding about FS and their possible participation in the reproductive process and other unknown effects in different phenomena related with the changes in the chromosomal damage rate of the normal populations.

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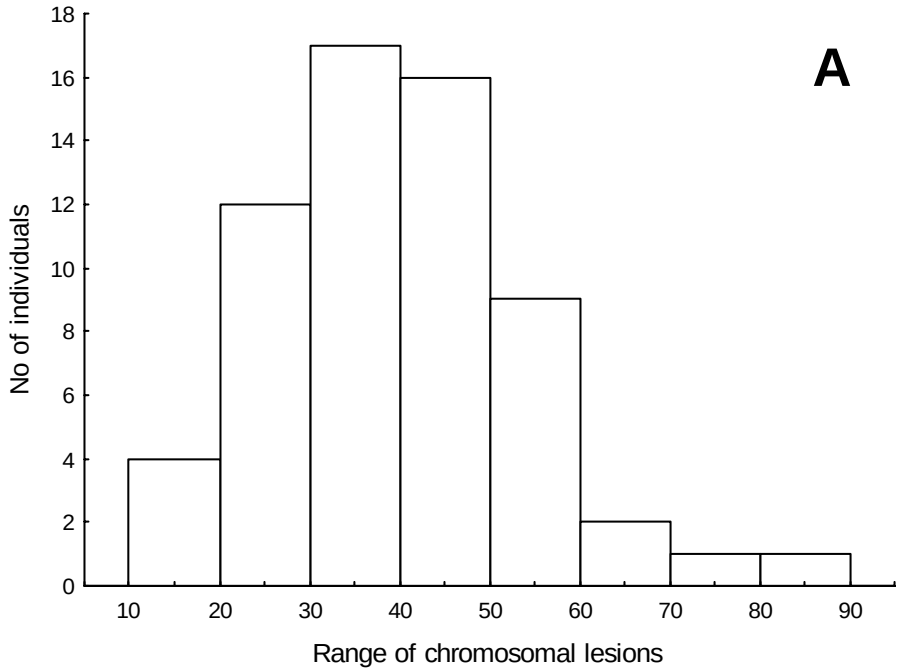
TABLES AND FIGURE LIST

Sample	No. of individuals studied	No. of cell studied	Total number of gaps and breaks	Mean No. (± SD) of gaps and breaks per individual per 100 cells	Mean No. (± SD) of gaps and breaks per cell
Healthy	100	10000	2025	20.25±7.70	0.202±0.770
Infertile	62	6.200	2565	40.44±14.5	0.404±0.145
Total	162	16200	4585	28.30	0.303

Table 1. Means and SD of chromosome breakage in each sample set.



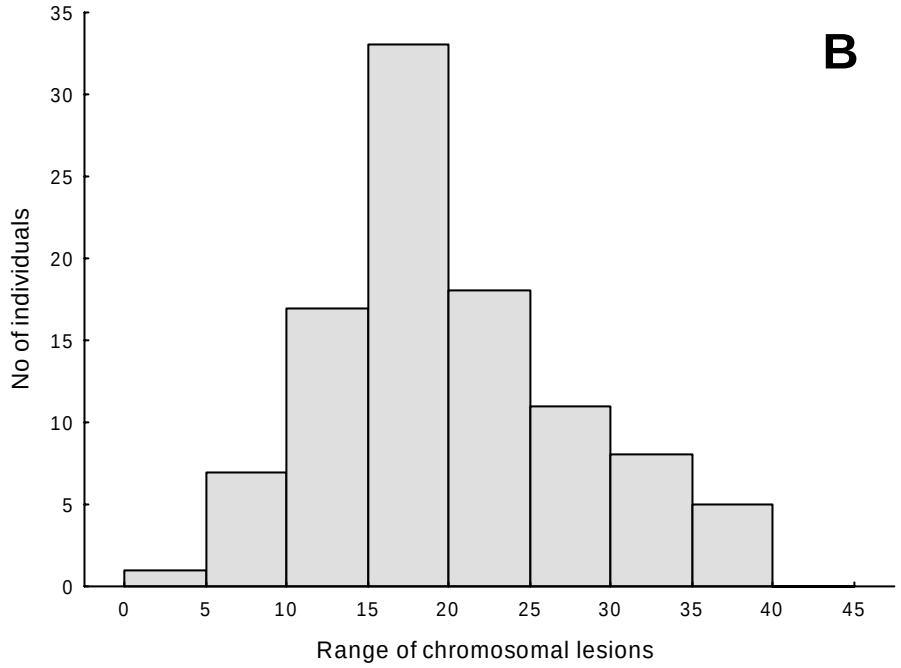


Figure 1. Distribution of chromosomal Breaks or gaps number in an infertile (A) and healthy (B) samples

Band	Healthy sample	Infertile sample
1p21	5	3
1p31	28	34
1p36	8	10
1q42	6	7
2p16	9	9
2q13	3	6
2q22	4	6
3p14	617	829
3p24	2	6
3q25	4	8
4q27	2	6
4q31	10	16
5p14	7	4
5q15	3	7
5q31	9	4
5q35	2	6
6q25	19	31
6q26	97	185
7q21	3	6
7q22	3	6
7q31	4	6
7q32	12	17
8q21	1	6
8q22	7	11
8q24	5	7
9q12	10	13
9q32	7	9
9q34	6	8
10q22	4	11
10q25	6	8
11p14	6	8
11p15	7	8
11q13	9	5
11q14	3	10
11q23	4	7
12q13	4	6
12q21	10	10
12q24	7	10
13q21	10	12
13q32	10	11
14q23	4	6
16q22	4	6
16q23	17	24
19p13	5	7
Xp22	11	11
Minimal value	5	6

Table 2. Number of gaps and breaks in each sample for the statistical significant fragile sites.

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