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Intermediate and normal sized CGG repeat on the *FMR1* gene does not negatively affect donor ovarian response

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BACKGROUND: Fragile X syndrome is associated with low ovarian reserve and poor ovarian response. The aim of this study was to investigate whether CGG repeats on the fragile X mental retardation 1 (*FMR1*) gene have predictive value for ovarian response to stimulation with gonadotrophins and for clinical outcome in our oocyte donation program.

METHODS: Oocyte donor candidates were selected according to Instituto Bernabeu oocyte donation program requirements. Fragile X genetic screening was performed in 204 oocyte donors, defining 141 controls and 63 cases: 35–39 repeats ($n = 34$), 40–45 ($n = 12$) and >45 ($n = 17$). All the patients underwent ovarian stimulation using a GnRH antagonist protocol and received a GnRH agonist trigger. The main factors used to measure outcome were oocyte yields, days of stimulation, gonadotrophin dosages, biochemical pregnancy, ongoing pregnancy and miscarriage rates.

RESULTS: No differences between the study group and controls were reported in oocyte yields (17.5 versus 18.9) or days of stimulation (11.40 versus 9.82). The control group used significantly more gonadotrophin (2212 versus1850 IU) than the study group. Clinical outcome was not affected by the CGG repeats on the *FMR1* gene in oocyte donors.

CONCLUSIONS: No negative effect was observed for intermediate-sized CGG repeats on ovarian stimulation and clinical outcome using a non-confounding model of oocyte donation. These results disagree with previous studies performed on infertility patients. Owing to the present study, fragile X genetic screening should not be considered for prediction of response to ovarian stimulation.

Key words: CGG repeats / *FMR1* gene / fragile X / ovarian response

Introduction

Fragile X syndrome (FXS) is not only the most common cause of inherited mental retardation, but allelic forms of *FMR1* could cause low ovarian response and premature ovarian failure (POF) as well. This disorder is associated with a dynamic CGG expansion in the *FMR1* gene located on the X chromosome. The full mutation that consists of alleles with >200 CGG repeats has a negative influence on transcription that results in the silencing of the fragile X mental retardation 1 (*FMR1*) gene with the consequent absence of the FMR1 protein (FMRP) (Bardoni and Mandel, 2002). Premutation alleles range from 55 to 199 CGG repeats and are associated with Fragile X Tremor/Ataxia Syndrome and Fragile X-associated primary ovarian insufficiency (FXPOI) (Wittenberger *et al.*, 2007). Women carrying a premutation are not affected by FXS but are at risk of transmitting the disease to their offspring since the premutation CGG segment is unstable and may expand and result in a full mutation (Visootsak *et al.*, 2005). The intermediate range consists of alleles of 45–54 repeats that are clinically uninvolved, although may be unstable when transmitted over generations (Nolin *et al.*, 2003).

The general population distribution shows a prominent peak between 29 and 30 triple repeats (Fu *et al.*, 1991). This distribution suggests different molecular mechanisms in the CGG behavior. Expansion of the (CGG)n element to full mutation generally leads to transcriptional silencing. Among premutation carriers, FMRP levels are gradually reduced with increasing repeat numbers, despite elevated *FMR1* mRNA levels, suggesting that translation is impeded within the premutation of intermediate or normal range. From these data,

30 repeats have been suggested as the switching point between positive and negative translation effects of the *FMR1* gene product (Chen *et al*., 2003) and the ovarian dysfunction in <200 CGG repeat carriers is not caused by the absence of FMRP (Streuli *et al*., 2009).

An excessive number of CGG repeats on the *FMR1* gene predisposes to POF (Wittenberger *et al*., 2007). The prevalence of POF in women who carry a premutation is estimated between 13 and 26% (Sullivan *et al*., 2005). Carriers of premutations have been identified in 13% of women with familial POF (Bussani *et al*., 2004). For intermediate carriers, the main drawback in showing an association between POF and CGG repeats is the cut-off for this range. Two studies showed an association between POF and intermediate-sized repeats, although the definition of intermediate sizes was different: 35–54 repeats (Bretherick *et al*., 2005) and 41–58 (Bodega *et al*., 2006). A recent study considers intermediate sized 35–58 repeats and concludes that intermediate sizes should not be considered a high-risk factor for POF (Bennett *et al*., 2010).

In the normal range (<45 repeats), milder forms of POF have also recently been reported (Streuli *et al*., 2009; Gleicher *et al*., 2009a). Early follicular phase serum FSH concentrations were found to be significantly higher in patients with POF and controls with more than 30 CGG repeats when compared with their counterparts with fewer than 30 CGG repeats (Chatterjee *et al*., 2009). Gleicher *et al.* (2009a) reported a direct statistical association between the number of CGG repeats (at 35–55) and ovarian reserve, as reflected by anti-Müllerian hormone (AMH) levels. Gonadotrophin dosages in controlled ovarian stimulation usually increase with decreasing ovarian reserve. According to the effect of CGG repeats on ovarian senescence, we would expect a correlation between the number of CGG repeats and the yield of the stimulation. To date only one work has been published concerning ovarian stimulation and *FMR1* expansion in infertility patients (Gleicher *et al*., 2009b). This work reported differences in gonadotrophin dosages and numbers of oocyte retrieved in patients carriers of >35 CGG repeats.

In order to show a correlation between CGG repeats and controlled ovarian stimulation, we proposed evaluating ovarian stimulation in a non-confounding model with patients from an oocyte donation program because oocyte donors are young or fertile women with normal ovulation, and there is minimal variability in oocyte and embryo quality. The goal of this study was to investigate whether CGG repeats on the *FMR1* gene have predictive value for ovarian response to stimulation with gonadotrophins, oocyte yield, days of stimulation or cycle outcome in a large oocyte donor program.

Materials and Methods

Study and control populations

Oocyte donations are the best model to evaluate the determinants of implantation for several reasons. First of all, oocyte and embryo quality vary minimally, as donors are young women with normal ovulation. Secondly, the preparation of the endometrium is similar, as all recipients receive the same hormone replacement protocol.

The selection and recruitment of our donors is carried out in our clinic following strict quality criteria, including an extensive chromosomal and genetic evaluation. All donors met the legal requirements in Spain (Spanish Law 14/2006). They must be aged between 18 and 35 years, healthy, with no family history of hereditary diseases. The donors undergo a complete gynecological examination, karyotype and screening for infectious diseases such as HIV, hepatitis B and C, gonoccocia and syphilis. In addition to the legal requirements, we perform genetic screening for cystic fibrosis and α and β thalasemia. Since 2008 we have performed fragile X genetic screening routinely on all of our oocyte donors, as a part of our extensive donor evaluation prior to being enrolled in the program.

In this study, we included the results of fragile X genetic screening from 204 oocyte donors (141 control and 63 study groups). Women who carry <35 CGG repeats belong to the control group. The study population was divided into three groups according to ACOG criteria: women in the considered normal range of 35–39 repeats ($n = 34$) and women in the intermediate range were subdivided in 40–45 ($n = 12$) and >45 ($n = 17$) repeats.

All the subjects included in the study gave their informed consent to collect peripheral blood samples suitable for molecular analysis. This study involved only retrospective analysis of anonymous medical records and was approved by the Instituto Bernabeu Institutional Review Board.

Genotyping

DNA was isolated from pheripheral blood lymphocytes according to the manufacturer's instructions (Wizard® Genomic DNA Purification Kit, Promega, Madison, WI, USA) and stored at 4°C. The *FMR1* CGG repeat length size was determined as previously described (Ennis *et al*., 2006) using PCR amplified with fluorescently labeled primers (Fu *et al*., 1991) and sized by capillary electrophoresis on an ABI 310 instrument (Applied Biosystems, Madrid, Spain). When only one allele size was identified, Fragile X PCR® from Abbot Company was performed in accordance with the published recommendations by commercial assay. Therefore, females with a single allele by conventional PCR could either be homozygous for that allele or have a cryptic expansion that can only be detected by specific assays. The allele with lower triplet repeats was designated allele-1 and the one with higher number as allele-2 (Gleicher *et al*., 2009a). For this study, allele-2 was used to assess associations with ovarian response parameters and cycle outcome.

Ovarian stimulation and oocyte retrieval

After following the Spanish Fertility Act requirements, all the patients received a controlled ovarian stimulation protocol with tailored doses of urinary FSH, (Fostipur, Angelini, Spain). Gonadotrophin stimulation started from Day 2 of menstrual cycles, with doses varying between 150 and 225 IU/day depending of the age of the donor, BMI and antral follicle count. The GnRH antagonist, cetrorelix (0.25 mg/day; Cetrotide; Merck-Serono), was introduced according to a multiple-dose, flexible protocol. In all cases, triggering was exclusively performed with 0.2 mg of subcutaneous triptorelin (Decapeptyl; Ipsen Pharma). Ovarian response was monitored by transvaginal ultrasound and plasma estradiol concentrations. Oocytes were aspirated 36 h after analog administration by transvaginal, ultrasound-guided needle aspiration under sedation. Sperm and oocyte preparation, fertilization, embryo culture and transfer were performed according to IVF laboratory guidelines.

Recipient protocol

Recipient women carried out a standard protocol. The protocol for steroid replacement included pituitary desensitization with a single i.m. ampoule administration of 3.75 mg of triptorelin (Decapeptyl depot 3.75; Ipsen Pharma) in the midluteal phase of the menstrual cycle. Oral estradiol (E_2) valerate (Progynova, Schering) or E_2 patches releasing 50 μg daily (Dermestril 50; Rottapharm-Madaus) was used in a progressively increasing dose regimen for the endometrial preparation. Patients received up to 6 mg E_2 valerate a day or 3 patches every other day and

the duration of the treatment varied in accordance with the availability of a phenotype matched donor, ranging from 10 to 29 days. After 13 days of E_2 valerate administration, endometrial thickness and pattern were tested. If a three-layer pattern was observed in a $\geq$7 mm endometrium, the early-mentioned dose of E_2 therapy was continued at least until the pregnancy test was performed 2 weeks afterwards. If the endometrium was not seen to be sufficiently developed, doses of E_2 valerate were increased to 8 mg/day or four patches. From the day of oocyte retrieval, 600 mg of micronized progesterone (Utrogestan; Seid) was administered vaginally daily until the pregnancy test was performed.

Statistical analysis

All the statistical assessments were made based on the allele-2 associations. Values are presented as means $\pm$ SD for continuous data and percentages for categorical variables. Continuous data were analyzed by Student's *t*-test and one-way analysis of variance with *post hoc* Tukey's multiple comparison test, where appropriate. For categorical variables, differences between groups were tested by the χ^2 test. A $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS software (SPSS, Inc., Chicago, IL, UISA).

Results

FMR1 allele size

All female candidates to the Instituto Bernabeu oocyte donation program have to pass a psychological evaluation and a gynecological check-up. After this, infectious and genetic studies are carried out in order to assure the health of the offspring. Karyotype, screening for alpha and beta thalasemia, cystic fibrosis and fragile X genetic screening, are part of our strict selection and recruitment protocol and consequently have been performed on all of our oocyte donor candidates. For this study, we included the results of the *FMR1* CGG repeat counts obtained from 204 oocyte donors. Figure 1 shows the distribution of CGG repeats numbers on allele-2 in this study and the shift from the distribution of Fu's 1991 unselected population (data not shown). The most common allele is 30 repeats. In the study group, the mean allele-2 size is 42 repeats with a range of 35–62 repeats. In the control group, the mean allele-2 size is 29 repeats, with a more narrow range of alleles from 17 to 34 repeats. Two patients from the study group were rejected because they carried CGG repeats size above 55 (56 and 62) that shift into the premutation range.

Ovarian stimulation

Table I summarizes the different groups of ovarian stimulation parameters. No differences were observed in donor age (25.4 $\pm$ 3.5 versus 25.2 $\pm$ 3.4 years) and number of oocytes retrieved (17.5 $\pm$ 8.0 versus 18.9 $\pm$ 8.2) between control and study groups (respectively) and subgroups. The age from 35 to 39 repeats group showed a significance difference from the control group (23.6 $\pm$ 2.9 versus 25.4 $\pm$ 3.5 years), and this difference could explain why this subgroup used less gonadotrophin than the control group.

Gonadotrophin doses were related to the number of CGG repeats. Women from the control group used significantly more gonadotrophin (2212 $\pm$ 655 IU) than the study group (1850 $\pm$ 361 IU). Further, the control group showed higher gonadotrophins than all subgroups, although this difference did not reach significance in the 40–45 subgroup (Table I). The number of days of stimulation was also associated with the number of CGG repeats. The days of stimulation for women from control group were 11.4 $\pm$ 2.1 compared with 9.8 $\pm$ 1.4 for women from the study group, and only subgroups 35–39 (9.6 $\pm$ 1.5) and 40–45 (9.8 $\pm$ 1.3) showed a significant difference from the controls. These results disagreed with previous studies performed on infertility patients.

Cycle outcome

Oocyte donation treatment outcomes are given in Table II. We compared the results between the control group (<35) and the study group (>35) and subgroups (35–39, 40–45, >45). Overall, 204 cycles were considered for this study and no significant differences in cycle outcome were observed between the control group and

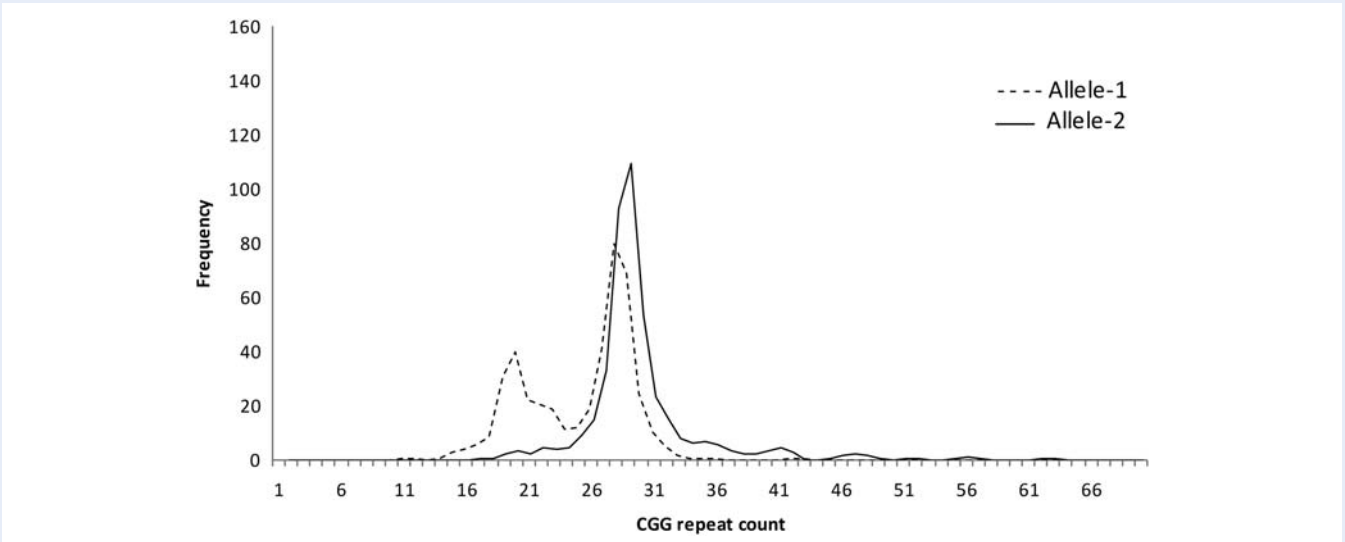

Figure 1 Distribution of CGG triplet repeats expansions on the *FMR1* in the whole oocyte donor study population.

Table I Comparison of ovarian response between groups.

CGG repeats	Control <35	Total study >35	35–39	40–45	>45
Number of patients	141	63	34	12	17
Donor age (year)	25.4 ± 3.5	25.2 ± 3.4	23.6 ± 2.9[a]	26.0 ± 2.7	28.0 ± 2.8
No. of stimulation days	11.4 ± 2.1	9.8 ± 1.4[a]	9.6 ± 1.5[a]	9.8 ± 1.3[a]	10.4 ± 1.2
Gonadotrophin dosage (IU)	2212 ± 655	1850 ± 361[a]	1846 ± 348[a]	1959 ± 348	1779 ± 411[a]
No. of retrieved oocyte	17.5 ± 8.0	18.9 ± 8.2	19.6 ± 9.1	20.0 ± 8.0	17.5 ± 6.5

[a]Denotes a statistically significant difference ($P < 0.05$).

Table II Comparison of cycle outcome between groups.

CGG repeats	Control <35	Total study >35	35–39	40–45	>45
Number of patients	141	63	34	12	17
Recipient endometrial thickness (mm)	9.4 ± 2.0	9.2 ± 1.7	9.4 ± 1.8	8.7 ± 1.7	9.2 ± 1.7
Transferred embryos	1.9 ± 0.5	2.0 ± 0.5	2.0 ± 0.6	1.9 ± 0.5	2.1 ± 0.5
Biochemical pregnancy rate (%)	57.4	61.9	61.8	66.7	58.8
Clinical pregnancy rate (%)	46.8	54.0	52.9	66.7	47.1
Implantation rate (%)	36.5	32.8	34.6	39.1	25
Miscarriage rate (%)	25.8	11.8	5.3[a]	14.3	25.0

[a]Denotes a statistically significant difference ($P < 0.05$).

study group, or between different subgroups. No differences between the control and study groups were observed in recipient patient endometrial thickness (9.4 ± 2.0 versus 9.2 ± 1.7 mm) (Table II), days of hormone replacement therapy (21.4 ± 6.3 versus 19.4 ± 5.3), fertilization technique or the day of embryo transfer (data not shown). We obtained more embryos in the study group compared with the control group (7.1 versus 6.2, respectively). These results did not affect the clinical outcome. In fact, there were no significant differences with respect to biochemical pregnancy (57.4 versus 61.9%), miscarriage (25.8 versus 11.8%), ongoing pregnancy rate (46.8 versus 54.0%) or miscarriage rate (25.8 versus 11.8%). Between the control groups and subgroups, the results did not show any significant differences, only the miscarriage rate between the control and the 35–39 CGG repeats groups was significantly different (25.8 versus 5.3%, respectively).

Discussion

To our knowledge, these data show for the first time the relationship between normal and intermediate-sized CGG repeats on *FMR1* gene and ovarian stimulation and clinical outcome using a non-confounding model of oocyte donation. Our data suggest that ovarian stimulation is not negatively affected by CGG repeats in the normal or intermediate range. The number of oocytes yielded and the clinical outcome are not associated with CGG repeats. In contrast to previous studies, lower gonadotrophin doses and fewer days of stimulation are needed for women with 35–45 CGG repeats.

The main reason for investigating the CGG expansion on the *FMR1* gene has historically been diagnosis of neurological conditions, which have been associated with excessively high triple repeat expansions of premutation and full mutation sizes (Gleicher *et al.*, 2009c). Therefore, the classification of CGG repeats ranges is based on a neurological risk screening and has no relevance to other potential risks associated with CGG repeat number such as POF and ovarian function. Recent publications show a statistically significant correlation between ovarian reserve and CGG repeat number between 35 and 50 repeats (Gleicher *et al.*, 2009d), this is the normal range associated with neurological risk. AMH levels are representative of small, growing follicles and the number of follicles remaining in ovaries has been suggested to be the most reliable indicator of ovarian reserve (Faddy, 2000). AMH correlates throughout the entire spectrum of triplet repeats on the normal and intermediate range (Gleicher *et al.*, 2009a). Other important data provided here are the distribution of triple CGG repeats (Fig. 1) in the general population that noted a normal distribution range of approximately 28–33 with a large majority of individuals between 29 and 30 repeats. Since the distribution of 29–30 repeats correlates with the switching point between positive and negative translation effects on FMRP (Chen *et al.*, 2003), this mechanism could explain consequences on ovarian function. The U-shaped risk curve for FXPOI in the permutation size range could be explained by the effect of the number CGG repeats in FMRP translation. Translation of the gene product may, for example, suppress proliferative abilities of smaller follicles. Altogether, the normal (<35 repeats) and intermediate ranges are considered normal in fragile X testing but they may be abnormal in terms of ovarian function.

Successful outcomes following assisted reproductive technology (ART) are largely dependent on the patient's response to controlled

ovarian stimulation. There are several factors that can predict the ovarian response and therefore the likelihood of success following ART. Ovarian reserve is probably the most important factor in determining success rates after IVF. Gonadotrophin dosage for ovarian stimulation usually increases with decreasing ovarian reserve, though it is unknown whether such increases in stimulation improve oocyte yield. A recent report showed that increasing gonadotrophin use and decreasing oocyte retrieval are, therefore, expected beyond age 38 years and with triplet CGG numbers beyond 35 (Gleicher *et al.*, 2009b). Notwithstanding, this research was carried out on infertile women with ovarian dysfunction and we wanted to investigate this finding in normal populations, such as oocyte donors.

Oocyte donors are a fertile population where you can check any reproductive parameter without confounding. Oocyte donation is the best model to evaluate the determinants of stimulation and embryo implantation potential. On the one hand, donors are young women of similar age with normal ovarian function and, in our oocyte donation program, with previous fertility. On the other hand, the preparation of the recipient endometrium is similar, as all recipients receive the same hormone replacement therapy.

Our data from our oocyte donation program suggest that ovarian stimulation is not negatively affected by CGG repeats within the normal and intermediate range. The number of oocytes yielded and the cycle outcome are not influenced by the CGG repeats. Unlike previous studies, less gonadotrophin and days of stimulation were needed for women with 35–45 CGG repeats. This finding could be explained by a slightly significant difference in age from the control group and the 35–39 repeats study subgroup. Otherwise, no negative effect could be shown in patients with >35 repeats on ovarian stimulation and cycle outcome. Therefore, such alleles are not associated with disadvantageous or unsuccessful ART.

From the point of view of neurological outcomes, differences in opinion exist between ACOG (ACOG Committee Opinion, 2010) and others (Wittenberger *et al.*, 2007) whether the intermediate stage of triple repeats should start at 40 or 45 repeats, but regarding ovarian senescence, different reports showed an increased risk for milder forms of POF and >35 CGG repeats that could affect ovarian stimulation. From our data, no differences in ovarian response could be shown in a fertile population with CGG repeats in the normal and intermediate range, regardless of whether the cutoff of 40 or 45 repeats was used.

Previous observations point towards the future utilization of *FMR1* gene testing as a diagnostic fertility test of ovarian senescence. However, from our data normal and intermediate-sized CGG repeat alleles should not be considered as a predictor of response to ovarian stimulation. Notwithstanding, we emphasize the need to carry out FXS screening in all the candidates for oocyte donation and patients with POF in order to avoid expansion to full mutation in the offspring.

In conclusion, our study suggests that ovarian stimulation is not affected by CGG repeats on *FMR1* in the normal and intermediate range in fertile women, providing no predictive value for ovarian response to stimulation with gonadotrophins. However, *FMR1* testing could be involved in the comprehension of the etiology of ovarian insufficiency and in the prevention of transmission of fragile X, taking into account CGG pathological ranges.

Authors' roles

B.L., J.Gu. and J.L. involved in the study design. J.Gi. participated in sample collection and oocyte donors recruitment. B.L., J.A.O. and R.M. performed laboratory experiments. B.L. and J.Gu. involved in analysis. B.L., J.Gi. and J.T. involved in manuscript drafting. B.L., J.Gu., J.A.O., R.M., J.L., J.Gi., J.T and R.B. participated in critical discussion.

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Conflict of interest

None declared.

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