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Do IGF1 Polymorphisms Really Matter for Fertility?

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Abstract

Our aim was to investigate the possible association between *IGF1* gene, fertility and implantational success. A case-control study has been conducted with a patient sample of 49 unrelated Brazilian couples who underwent assisted techniques due to idiopathic infertility, and 123 control fertile couples. After DNA extraction from the peripheral blood by salting-out method, exons 3, 4 and 5 of *IGF1* gene, as well as their flanking regions, were amplified by PCR. Genetic variability was evaluated by Sequence Base Typing (SBT). Possible associations were estimated by binary logistic regression models. Thirteen allelic variants were found in *IGF1* gene, but only three of these loci were considered polymorphic. These SNPs were rs5742620 (C > A), rs2072592 (G > A) and rs11111267 (T > C), in intronic regions. Although this study could not find an association between *IGF1* and infertility, a possible association cannot be ruled out, due to the complex network regarding reproduction process. The higher frequency of rs11111267(C) in male patients is very suggestive that this SNP may play a role in male fertility and deserves further investigation. Genetic variation in intronic regions of the *IGF1* might alter the gene expression level which may impact gametogenesis, embryo implantation and zygote development.

Keywords: Assisted Reproduction; IGF1; Infertility; Polymorphism

Introduction

It is estimated that 10 to 15% of population in reproductive age is infertile, being men and women equally affected. Infertility is defined as the inability of a couple to conceive after 12 months or more of unprotected and regular intercourse. Among these couples, the infertility cause remains unknown for 15 to 20% of the cases. Demand for assisted reproduction technology (ART) increased in the last few decades. Conventional *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are used as standard fertilization techniques in ART for female and male factor infertility, respectively, and they have improved artificial conception and live birth rates for women younger than 35 years old [1]. Although there is a clear improvement in the success rates of ART cycles, due to better superovulation protocols, embryo culture media and embryo transfer techniques, the pregnancy rates still vary between fertility centers. Despite of some known factors that could explain this variation in part, such as women's age and the number of retrieved oocytes after superovulation [2], there are variables that remain unknown. An important group of growth factors that is constantly being studied and associated with reproductive success since gametogenesis and embryo implantation in the uterus up to intrauterine growth is the insulin-like growth factor (IGF) axis, highlighting IGF-1. Growth factors are a group of signaling proteins, capable of inducing cellular proliferation and differentiation [3].

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IGF-1 binds to its cognate receptor (IGF1R), which contains an intracellular tyrosine-kinase domain and is localized in cytoplasmic membrane [4]. This interaction between IGF-1 and IGF1R elicits an intracellular signaling cascade through the cytoplasm to the nucleus, where transcriptional factors activate or block the transcription of specific genes, altering their expression levels [3, 4]. IGF-1 has been associated with mitogenesis, proliferation, cell growth, differentiation, angiogenesis and cell survival [3]. These activities are of utmost importance for reproduction, from gametogenesis and embryo implantation to the development of the zygote. The effect of IGF-1 on reproduction was previously studied in mammals such as mice [5, 6], bovines [7], as well in humans [8-12]. Particularly in human reproduction studies, IGF-1 have been positively associated with male and female gametogenesis [8,10]. In spermatogenesis, IGF-1 serum levels were lower in infertile men when compared with fertile men; moreover, this differential expression was correlated with semen quantity and sperm quality [8]. On the other hand, in oogenesis IGF-1 is involved in follicular growth and maturation [10]. After fertilization, IGF-1 was associated with increased embryo growth and development to blastocyst stage, while it decreased cell death by apoptosis [12]. IGF-1 serum levels in women prior to IVF cycle were correlated with the likelihood of a live birth, where higher median concentrations of this growth factor leaded to better outcomes [11]. A prospective study have verified the relationship between IGF-1 levels in follicular fluid and embryo quality, fertilization rates, and confirmation of clinical pregnancy among women undergoing IVF; the retrieved data suggests that women with higher levels of IGF-1 presented better IVF outcomes [9]. Although it is not hard to find studies concerning IGF-1 protein levels and reproduction [5, 6, 8-11, 13-15], studies investigating potential allelic variants and polymorphisms in the gene encoding IGF-1 are scarse. It was described a case report of IGF-1 deficiency associated with severe phenotypes such as pre- and post-natal growth retardation, delayed psychomotor development and sensorineural deafness possibly caused by a transversion T>A in the 3'untranslated region (3'UTR) of IGF-1 gene (*IGF1*) [16]. It was suggested that this point mutation could disrupt the polyadenylation site in exon 6, leading to altered mRNA processing and low IGF-1 circulating level [16]. Another study reports a deletion of 192bp in promoting region of *IGF1*, which could reduce the plasma IGF-1 concentration and consequently impair post-natal growth [17]. *IGF1* is localized in chromosome 12q22-23, and it contains six exons and five introns along more than 85kb [16-18]. Its structure is highly complex and it possesses multiple transcription initiation sites, alternative splicing and different polyadenylation sites, resulting in distinct RNA transcripts [19]. However, only exons 3 and 4 encode the mature peptide of IGF-1; hence, they are necessarily transcribed [19, 20]. Exon 5 carries most of the variability of *IGF1* and, when transcripted in the mRNA, it codifies the pro-IGF-1 called IGF-1Eb; on the other hand, when exon 6 is transcripted it originates IGF-1Ea. Still, another alternative splicing in RNA transcripts that carries regions coded by both exons 5 and 6 leads to the pro-IGF-1Ec [16, 19, 20]. IGF-1Ea is mainly involved in post-natal growth, whereas IGF-1Eb is related to pre-natal development; the role of IGF-1Ec is not yet known [16]. Following translation of mRNA, posttranslational modification occurs to generate heterogeneous products [18]. Point mutations in intronic or exonic regions of *IGF1* may affect RNA transcription or processing, as well as the bioactivity of IGF-1 Although IGF-1 expression is minimally regulated by external factors to the fetus, being mostly under genetic control [21], there are no studies exploring *IGF1* polymorphisms and IVF outcomes. Therefore, we aim to investigate if there is an association between genetic variability of exons 3, 4 and 5 of *IGF1*, as well as their flanking regions, fertility and implantational success in couples undergoing IVF/ICSI.

Materials and Methods

Subjects

The patient group consisted of 49 Brazilian unrelated couples undergoing ART by IVF/ICSI due to idiopathic infertility. The couples were recruited from Clínica Conceber, a reproductive medicine center located in Curitiba city, Parana state, in southern Brazil. All couples were previously submitted to a standard screening program (sperm analysis and evaluation of hormonal status) before starting treatment. Exclusion criteria comprise men diagnosed with obstructive azoospermia and women over the age of 35 years old, with endometriosis, decreased ovarian function, anatomical abnormalities of the uterus, number of retrieved oocytes smaller than three after superovulation treatment, or FSH and estradiol levels higher than 12µL/mL and 80pg/mL, respectively. These exclusion criteria intended to prevent that a possible implantation failure could be due to lower responses of the couples to the IVF/ICSI treatment, apart from minimizing the patient group heterogeneity. Control group was comprised of 123 unrelated control fertile couples from Parana state, southern Brazil. All couples from control group had at least two children and had no history of miscarriages.

Ethical Approval

The Ethics Committee of Research in Human Participants of the Health Sciences/UFPR (CED/SD-UFPR) approved this study (CEP/SD-PB 2081319). All participants from both groups signed a free and informed consent form, and a personal and occupational questionnaire.

IGF1 Sequencing

EDTA blood samples were collected from all participants and genomic DNA was extracted using a salting-out

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procedure [22]. DNA amplification of exons 3, 4 and 5 of *IGF1* was performed by polymerase chain reaction (PCR) resulting in products of 351, 463 and 695 base pairs, respectively. Each exon was amplified in a distinct PCR. The amplified DNA fragments contained the complete exon sequence, but they also included an upstream and a downstream flanking intronic region. The following set of primers were employed in PCR reactions: exon 3 forward – 5' ATCGTGGGAGTCAATGCACT 3' and exon 3 reverse – 5' AGATACGGGCACTCATTCAGTT 3'; exon 4 forward – 5' TACAGACTCCGGGAGACATACT 3' and exon 4 reverse – 5' TATGGGGCAGGATTTCTGCTT 3'; and exon 5 forward – 5' ACCACTTGTTCTCAATGCA 3' and exon 5 reverse – 5' AGGGAATCTGGGAACTTCTA 3'. Amplification reactions were conducted using 50ng of genomic DNA in a final volume of 25µL with 1X PCR buffer (70mM Tris-HCl, pH 8.8, 20mM (NH4)2SO4), 1.5mM MgCL2, 0.2mM of each dNTP, 0, 2µM of each primer and 1.0 unit of Taq DNA polymerase Platinum (Invitrogen, Carlshad, CA, USA). For exons 3 and 4, the initial denaturation cycle was carried out at 94ºC for five minutes, followed by 35 cycles of 95ºC for 30s, 60ºC for 30s, 72ºC for 60s, and a final extension step at 72ºC for 7 minutes. Exon 5 was amplified by the same thermocycling conditions, with the exception of the annealing temperature, which was set as 56°C, instead of 60°C. Six microliters of PCR products were purified by enzymatic method using 10 units of Exonuclease I (United States Biochemical – USB, Staufen, Germany), 2 units of Shrimp Alkaline Phosphatase (SAP) and 1X SAP Buffer (United States Biochemical – USB, Staufen, Germany). The purification conditions were as follow: 37°C for 60 minutes for enzymatic treatment and 80°C for 15 minutes for enzymes inactivation. Purified PCR products were sequenced using ABI Prism Big Dye Terminator Cycle Sequencing Kit v.3.3 (Applied Biosystems, CA, and USA) and ABI Prism 3130 Genetic Analyzer (Applied Biosystems, CA, USA). The same primers employed in PCR amplification were used in the direct DNA sequencing reaction. The obtained sequences were aligned and compared with reference sequences for *IGF1* exons 3, 4 and 5, deposited in Ensembl browser, using SeqScape Software 2.7 version (Applied Biosystems, CA, and USA). Each detected allelic variant was individually noted and compared with genetic variants described in NCBI dbSNP.

Statistical Analysis

IGF1 allele and genotypic frequencies of all individuals were calculated by direct counting and Hardy-Weinberg equilibrium were evaluated using Arlequin 3.5.1.2 software [23] and $\chi 2$ tests [24]. Binary logistic regression models were employed to calculate the Odds Ratio (OR) and 95% confidence intervals (16) for assessment of the *IGF1* individual's and couple's combined polymorphisms contributions on fertility (patients *vs* controls). P-values ≤ 0,05 were considered statistically significant. Genotypic association of each variable was conducted under dominant and co-dominant models. Statistical analysis were performed using Stata software 14.2 version.

Results and Discussion

Sample Characterization

Of the 49 women submitted to IVF/ICSI, embryo implantation was successfully established in 41.46% (n=17), since beta hCG was detected in the bloodstream after embryo transfer to the uterus. However, two of these patients had spontaneous abortion. In the remaining 58.54% patients (n=24) the ART resulted in embryo implantation failure, once beta hCG was not detected after embryo transfer. Access to beta hCG detection exam for eight of the 49 women was not available. Mean age of female patients was 31.53 ± 2.39 years old. With stratification of patients based on beta hCG detection, mean age of success patients was 30.76 ± 2.16, while implantation failure patients mean age was 31.86 ± 2.40. Of all 49 TRA couples, 65.12% had already undergone to some assisted reproduction technique before. After ovarian stimulation, it was obtained an average of 10.03 ± 5.52 oocytes per patient. Fertilization procedures resulted on an average of 3.81 ± 2.19 live embryos, of which 2.22 ± 0.68 embryos were transferred to the uterus. On the other hand, mean age of women in control group was 23.91 ± 4.63 years old during their first pregnancy, and it was 29.21 ± 4.98 years old during the last pregnancy. All women had at least two natural and healthy pregnancies to term.

IGF1 Sequencing

It was observed 13 distinct allelic variants in the sequenced regions (data not shown). Three of them were located in the intron upstream exon 3, and one in the exon 3 itself. Two more variants were found in the intron upstream exon 4, and another two in exon 4. Finally, four allelic variants were noted in exon 5, and the last one was located in the intron flanking exon 5 downstream. Allele and genotypic frequencies were obtained by direct counting considering patient and control groups together and separately. All levels of analysis were in Hardy-Weinberg Equilibrium. Although 13 allelic variants were detected, only three *loci* presented the less frequent allele in a frequency of 1% or more, characterizing a single nucleotide polymorphism (SNP). These SNPs are rs5742620 (C > A), rs2072592 (G > A) and rs11111267 (T > C), which are all located in intronic regions. SNPs rs5742620 and rs2072592 are upstream exon 3 and exon 4, respectively, while SNP rs11111267 is downstream exon 5. Of these three SNPs, only for rs11111267 the minor frequency allele was present in a frequency higher than 10% both in the patient and in the control groups considered separately and together. Probably, none of these SNPs directly affect the composition nor the structure of IGF-1, since they are located in intronic

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regions and do not appear to influence mRNA processing with exons/introns inclusion or exclusion. However, SNPs in intronic regions have the potential to alter the activity of the protein product of a gene by modifying its gene expression, since an allelic variant may present lower or higher affinity to regulatory components, such as transcription factors, spliceosome components or miRNAs. Of these three SNPs, rs5742620 has already been associated with pre-eclampsia [25, 26]. The less frequent allele, which carries an adenine instead of a cytosine (C>A) would increase the risk of developing pre-eclampsia in either homo or heterozygous condition [25, 26]. This same allele was also associated with increasing risk of a growth restriction condition in which children are small for gestational age (SGA) [27]. Although there is no evidence of direct involvement of rs2072592 with gestation, this SNPs is involved with prostate development [28]. Yet, SNP rs11111267 has not been associated with any pregnancy complication nor disease or disorder related to male or female reproductive systems. The lack of nucleotide variations with relatively high frequencies in coding regions of a gene indicates its importance for the development and/or maintenance of the organism. Indeed, it has been demonstrated in murine models that not only IGF-1, but also IGF-2 are required for proper development in order to maintain normal embryonic growth rates, and the absence of IGF-1 or both growth factors may be lethal [13, 29, 30]. Besides partial lethality, mouse embryos carrying null mutations of *Igf1* that reached adulthood exhibited developmental delay, growth restriction and were infertile in both sexes [31] In humans, it was reported a case of a male patient who had a homozygous partial deletion in *IGF1* gene, leading to a truncated and inactive IGF-1 protein [32]. Although the patient presented severe prenatal and postnatal growth restriction, besides osteopenia, mental retardation and sensorineural deafness, infertility due to the null mutation in *IGF1* was not observed, despite a delay in puberty development [32]. Thus, the roles of IGF-1 on prenatal and postnatal development indicate a selective pressure that contributes to the maintenance of *IGF1* gene sequence in such conserved manner [33]. This conservation could be expected especially in exons encoding the mature polypeptide itself (exons 3 and 4) and regulatory regions such as promoters (exons 1 and 2), E-peptides (exons 5 and 6) and intronic regions that participate on regulatory events. Binary logistic models were applied to investigate possible associations between genetic variability in *IGF1* and fertility. Only the polymorphic variants were included in the logistic models, ie, *loci* rs5742620, rs2072592 and rs11111267. Several logistic models were tested considering women ages, menarche ages and different combinations of the three SNPs observed. Furthermore, the SNPs were evaluated considering three possible scenarios: (1) the female genotypes, disregarding a possible male contribution to the infertile phenotype; (2) the male genotypes, discarding female contribution to the phenotype; and (3) considering a combination of male and female genotypes. Binary logistic models were evaluated by their "Prob>chi^2" and "Pseudo R^2" values, and the most significant model is presented in Table 1.

As Prob>chi^2 = 0.025, the logistic model is considered significant. Moreover, the value of Pseudo^2 indicates that this model has an effect of approximately 5.62% on fertility. Of the independent variables, only women age can be considered significant [P = 0.008; OR = 1.100; CI = 95% (1.026-1.181)] within the defined parameters adopted in this work's methodology, even though female patients age were limited in up to 35 years old. Concerning genotypes, the co-dominant model lead to the construction of the most suitable model. Thus, the rs11111267 SNP in the male genotypes has an associated P value of 0.063, close to the threshold of significance, where cytosine (C) carrier genotypes in homozygosis or heterozygosis would exhibit a tendency to be present at a higher frequency in men of infertile couples. However, although the odds ratio for this variable is 2.149, the 95% confidence interval is between 0.9588 and 4.8163; hence, the fertility outcome for men carrying the SNP rs11111267(C) would not be predictable.

Table 1: Binary logistic regression model of the variable fertility, which compares couples in patient group and in control group.

LOGISTIC REGRESSION MODEL FOR THE DEPENDENT VARIABLE FERTILITY					
Pseudo R^2 = 0,0562			Prob > chi^2 = 0,025 *		
Independent variable	Odds Ratio	Robust standard error	Confidence Interval (CI = 95%)		P Value
Female age †	11,004	0,0396	10,256	11,808	0,008 *
Menarche age	10,739	0,1457	0,8231	14,011	0,599
Female genotype for SNP rs5742620	11,035	0,9273	0,2126	57,289	0,907
Male genotype for SNP rs2072592	10,604	0,9806	0,1731	64,962	0,949
Male genotype for SNP rs11111267	21,489	0,8848	0,9588	48,163	0,063
Model's constant	0,0042	0,0077	0,0001	0,1476	0,003 *

† For women in control group, it was considered their age when their youngest child was conceived, since they were still fertile. For women in patient group, it was considered their age when seeking ART, since they were already presenting infertility.
* P ≤ 0,05.

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In reproduction, IGF-1 is mainly studied in prenatal growth or at the maternal-fetal interface context (with blastocyst's development and / or embryo implantation in the uterus). Yet, even not significant (P = 0.063), the results of this work call attention to male infertility, considering that the genetic variation C to T in rs11111267, located in 3'UTR downstream exon 5 could affect the genetic expression of *IGF-1* related to spermatogenesis. Indeed, IGF-1 levels have already been related to spermatogenesis quantitatively and qualitatively, being positively associated with spermatogonia differentiation in primary spermatocytes, sperm motility and viability, and volume of semen produced in mice and humans [8, 14]. The almost inexpressive OR for women age was not a surprising data. According to American Society for Reproductive Medicine, the efficiency's peak of the female reproductive system occurs with a little over 20 years old, and decays relatively fast, mainly from the age of 35 years [34]. In an attempt to eliminate the influence of age on fertility, this study was designed to limit the age of patients within 35 years. However, the women of the patient group did not have their ages paired with those of the control group, which may have contributed to the differential age distribution. Yet, it should be noted that women in the control group may have remained fertile longer after the last gestation, and it is possible that ART patients was already infertile before seeking treatment. Despite this limitation, women age was evaluated in the best possible way. Unfortunately, some variables known to affect fertility, which may also influence the embryo implantation were left out in this study. Among these factors, we can mention the consumption of alcoholic beverages and anxiolytic drugs, besides excessively high or low weight. The inclusion of these variables would provide valuable information that could be used in the construction of logistic models that could suit better to this study. However, these factors were not investigated because of the difficulty in collecting this information in the case of the couples in the control group, since many of them had conceived the last child many years before the collection of blood samples, personal data and medical history, making it difficult to remember the information accurately.

Conclusions

There are several biological components interacting directly or indirectly for successful gestation, from the development of the female and male reproductive systems, to the formation and differentiation of progenitor cells in gametes, through fertilization of the zygote and its development in blastocyst, embryo implantation in the maternal uterus and placentation. In addition, several other anatomical, physiological and immune changes will allow the fetus to develop and grow. Genetic factors can affect male and / or female fertility; nonetheless, reproduction is an extremely complex process and not fully elucidated. Any molecule involved in one or more of these biological processes mentioned above has the potential to affect the fertility of an individual, with IGF-1 being only one of these components. IGF-1 is part of a group of proteins that acts together: the IGF axis, which is part of a regulatory network that contemplates several other components, such proteins of PI3K and MAPK pathways, growth hormone, estrogen and other molecules implicated in their regulatory network [4, 6, 35]. Furthermore, other biomolecules that do not interact with the IGF axis, at least apparently, have the potential to unbalance the finely controlled reproductive process. As examples, it can be listed (1) other growth factors such as transforming growth factor β, epidermal growth factors and vascular endothelial growth factor [3, 36-38]; (2) other hormones, such as progesterone and testosterone; (3) cytokines, such as the leukemia inhibitory factor and interleukins 6 and 11 [36]; and several other classes of carriers, receptors, cell signaling biomolecules and extracellular matrix components [3, 5, 6, 39]. Therefore, even an alteration that has the potential to significantly reduce or increase the level of one of these components may not have such a strong impact to the point of being easily observed in a relative small sample. Possibly, a decrease in the functional levels of a biomolecule in that network may be supplied, partially or totally, by another component that interacts or is present in a common biochemical pathway, as it was previously observed within IGF axis [30]. Nonetheless, it does not mean that some genetic variants are not important for the function of the coded protein, including allelic variants in intronic region, which may play a role in regulation processes. Hence, research regarding the reproductive process is quite complex, requires a large sample number and an effort to try to investigate not only a component but an integrated network of molecules that impact one or more phases of reproduction. Thus, bioinformatic approaches can be of great value for screening some biochemical components that may be interesting targets of study.

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Conflict of Interest Statement

The Authors declare that there are no conflicts of interest associated with this study.

Authors' Contributions

Veiga RN: Protocol/project development, Data collection or management, Data analysis, Manuscript writing/editing. Silva JS: Data collection or management, Data analysis,

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Manuscript writing/editing. Gelmini GF: Data collection or management, Data analysis, Manuscript writing/editing. Schuffner A: Manuscript writing/editing. Bicalho MG: Protocol/project development, Data collection or management, Data analysis, Manuscript writing/editing

Availability of Data and Material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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