Influence of Val16Ala SOD2 polymorphism on the in-vitro effect of clomiphene citrate in oxidative metabolism

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Abstract This study investigated the in-vitro antioxidant properties of the ovulation induction drug, clomiphene citrate, and assessed whether its effects are influenced by the Val16Ala polymorphism in the SOD2 gene, which encodes manganese superoxide dismutase enzyme. The investigation involved an in-vitro experimental protocol testing the effect of different concentrations of clomiphene citrate on antioxidant capacity, reactive oxygen species (ROS) production and peripheral blood mononuclear cell (PBMC) culture viability. A total of 58 healthy adult women were genotyped for the Val16Ala SOD2 polymorphism, and blood samples were collected to perform in-vitro experiments. ROS production and cytotoxicity assays were performed on blood and PBMC from carriers of different Val16Ala SOD2 genotypes. Clomiphene citrate exhibited antioxidant capacity and effects and decreased ROS production. The AA genotype displayed a more responsive antioxidant effect with clomiphene citrate treatment than other genotypes. AA and AV PBMC showed an increase in viability following treatment with 10 μmol/l clomiphene citrate when compared with control groups. The results suggest that clomiphene citrate exhibits antioxidant activity similar to that observed with other selective oestrogen receptor modulators, and the intensity of the effect appears to be SOD2 polymorphism dependent.

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KEYWORDS: antioxidant, clomiphene citrate, cytotoxicity, ovulation drug, pharmacogenetic, Val16Ala SOD2 gene polymorphism
Introduction

In the female reproductive system, reactive oxygen species (ROS) and antioxidants play a physiological role in folliculogenesis, oocyte maturation, luteal regression and fertilization (Agarwal et al., 2005). However, an increase in ROS production leads to oxidative stress, which has been associated with reduced oocyte fertilization (Jancar et al., 2007; Seino et al., 2002), and aberrant embryonic development, which leads to early embryo death (Johnson and Nasr-Esfahani, 1994; Matsuzuka et al., 2005). Recently, studies performed by Aurrekoetxea et al. (2010) have also suggested that oxidative stress affects the success of IVF.

These adverse effects may be caused by a chronic ROS imbalance resulting from environmental and/or genetic alterations in antioxidant enzyme activity. This is the case with superoxide dismutase enzymes (SOD), which play a crucial role in the protection against ROS-induced mitochondrial damage (Beyer et al., 1991). SOD dismutate the superoxide anion (\(O_2^-\)) in hydrogen peroxide (\(H_2O_2\)), which in turn is degraded by other enzymes in water. There are three SOD intracellular isoforms: the manganese-SOD (SOD2 or MnSOD), which is the mitochondrial isoform; and two copper/zinc-SOD isoforms (SOD1 and SOD3), which are the cytoplasmic and extracellular isoforms, respectively (Miao and St Clair, 2009).

Previous studies have demonstrated that SOD enzyme activity could be associated with oocyte quality and may influence the success of assisted reproductive techniques (Matos et al., 2009; Sabatini et al., 1999). This hypothesis was recently supported by a genetic study performed by Ruiz-Sanz et al. (2011), in which the SOD2 polymorphism that was analysed was characterized by a valine-to-alanine substitution (rs4880) at amino acid position 16 (Val16Ala), which results in three different genotypes – AA, VV and AV – and the Ala-MnSOD precursor generated 30–40% more of the active processed MnSOD homotetramer than the Val-MnSOD precursor. In this case, the Ala-MnSOD/mitochondria targeting sequence allows for efficient MnSOD import into the mitochondrial matrix, while the Val variant causes partial arrest of the precursor within the inner membrane and decreased formation of the active MnSOD homotetramer in the mitochondrial matrix (Shimoda-Matsubayasgi et al., 1996; Sutton et al., 2003). Ruiz-Sanz and et al. (2011) found that the AA-SOD2 genotype was a significant independent predictor of the likelihood of successful IVF pregnancy.

These results raised questions about the potential pharmacogenetic influence of the Val16Ala SOD2 polymorphism on the activity of target molecules used to induce ovulation or to perform ovarian reserve tests, such as clomiphene citrate. Clomiphene citrate is a non-steroidal selective oestrogen receptor modulator (SERM) used to induce ovulation in polycystic ovary syndrome (PCOS) and to perform ovarian reserve tests in assisted reproduction treatment (Badawy and Einashar, 2011; Steiner et al., 2005). However, despite the use of clomiphene citrate since its introduction in 1956 and the data from studies suggesting that other SERMs, such as tamoxifen and ramoxifen, have antioxidant effects (Cardoso et al., 2004; Obata, 2006; Wakade et al., 2008), studies investigating the influence of clomiphene citrate on oxidative metabolism are only just emerging.

Therefore, this study evaluated the potential antioxidant capacity and the in-vitro effect of clomiphene citrate on specific parameters of oxidative metabolism: blood ROS production and peripheral mononuclear blood cell (PBMC) culture viability. Additionally, this study evaluated whether these results could be directly influenced by the Val16Ala SOD2 polymorphism. This experimental model was chosen because the blood samples were easy to obtain from donors with different SOD2 genotypes and because PBMC express the oestrogen receptor alpha (ESR1) and beta (ESR2) proteins (Scariano et al., 2008) and, therefore, are potentially responsive to clomiphene citrate activity.

Materials and methods

All of the chemicals used for the biochemical and molecular analyses were purchased from Sigma (St Louis, MO, USA), Invitrogen (USA) or Cultilab Co (São Paulo, Brazil), unless otherwise stated.

Clomiphene citrate treatments

To test the potential in-vitro effects of clomiphene citrate on oxidative metabolism and lymphocyte proliferation, four different concentrations of clomiphene citrate (1, 5, 10 and 20 \(\mu\)mol/l) were used, as described in a previous study by Boström et al. (2000) performed in the rat theca/stroma. Clomiphene citrate was initially dissolved in 70% alcohol and then further dissolved in buffer or cell culture medium according the experimental protocol. All experiments were performed at least in triplicate.

Antioxidant capacity: radical-scavenging assay

The antioxidant capacity of clomiphene citrate at different concentrations was evaluated by monitoring its ability to quench the stable free radical di(phenyl)-(2,4,6-trinitrophenyl)iminoazanum (DPPH) (Choi et al., 2002). Ascorbic acid was used as a control antioxidant molecule at the same concentrations of clomiphene citrate. The scavenging ability of clomiphene citrate was expressed as a percentage of DPPH quenching.

Subjects and Val16Ala SOD2 polymorphism analysis

To test the effect of clomiphene citrate on ROS production and cell viability, blood and PBMC samples were collected from Caucasian women with different Val16Ala SOD2 genotypes. In a previous genetic study of the Val16Ala SOD2 polymorphism (Duarte et al., 2010; Montano et al., 2009; Tauffer et al., 2005) involving a population living in the southern region of Brazil, 58 young, healthy females were selected (26.4 ± 7.3 years old, menarche at 12.37 ± 3.48 years). Blood samples were collected from this group and the Val16Ala gene polymorphism was assessed by polymerase chain reaction and restriction fragment length polymorphism, described in detail by Tauffer et al. (2005).

Blood samples were collected by venipuncture, using Vacutainers (BD Diagnostics, Plymouth, UK) tubes with EDTA. Specimens were routinely centrifuged within 1 h of
collection for 15 min at 2500 g and aliquots of serum samples were stored at −20°C. Genomic DNA was isolated from peripheral blood leukocytes and from tissue samples using a DNA Mini purification kit (MoBio, California, USA). PCR amplifications were performed in a total volume of 50 µl containing 5 µl of ×10 buffer, 1 µl of 25 mmol MgCl2, 1.25 µl of 10 mmol dNTP, 0.5 µl of Taq polymerase (Gibco, NY, USA), 1 µl (40 pmol) of each primer, 3 µl (0.25 µg) of genomic DNA and 34.5 µl of ddH2O. The amplification primers (Gibco, NY, USA) for a 110-bp fragment of the human MnSOD gene were 5’-ACCAGCAGGCTGCGGCGGG-3’ (sense strand) and 5’-CCGGTTGATGTGAGGTTCCAG-3’ (antisense strand) with thermocycler parameters comprising an initial cycle of 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 61°C for 1 min and 72°C for 1 min. The final cycle was followed by an extension period of 2 min at 72°C. The PCR product (10 µl) was digested with HaeIII (15 U, 37°C, 6 h; Gibco, NY, USA). Digested products (23 and 85 bp) were visualized on a 4% agarose gel (Amersham Biosciences, Sweden) stained with ethidium bromide. A mutation was introduced by a primer mismatch to create a restriction site for HaeIII in the −9 codon and the following genotypes were observed: −9 Ala/Ala (23 and 85 bp); −9 Ala/Val (23, 85 and 110 bp); and −9 Val/Val (110 bp).

The SOD2 genotype frequencies were the following: AA, 25.9%; VV, 27.6%; and AV, 46.6%. The calculation of possible deviation from the Hardy–Weinberg equilibrium, which was produced by a primer mismatch to create a restriction site for HaeIII stained with ethidium bromide. A mutation was introduced by a primer mismatch to create a restriction site for HaeIII in the −9 codon and the following genotypes were observed: −9 Ala/Ala (23 and 85 bp); −9 Ala/Val (23, 85 and 110 bp); and −9 Val/Val (110 bp).

The SOD2 genotype frequencies were the following: AA, 25.9%; VV, 27.6%; and AV, 46.6%. The calculation of possible deviation from the Hardy–Weinberg equilibrium, which was used to assess the chi-squared goodness-of-fit, showed that the samples were in genetic equilibrium. From these patient samples were excluded patients who were smokers, obese, or using chronic medication, vitamin supplements or hormonal contraceptives. Patients included in the study had no cardiovascular medical history, no hypertensive disorder and no metabolic diseases or other morbidity that could affect the results. The research study described here is relevant.

Because a previous study performed by Montagner et al. (2010) showed an association between the Val16Ala polymorphism and dietary behaviour, this study selected volunteers with similar lifestyle behaviours regarding diet and physical activity to avoid possible environmental influences. Additionally, volunteers were asked to avoid consuming antioxidant-containing food 24 h before blood collection. The foods that were not consumed by the volunteers included salads, fruits and juices (natural or manufactured). Three subjects of each genotype, who were considered to have the ideal profile, were selected as blood donors for the biological assays that were performed in the study.

The blood samples were collected by venous puncture into grey and red top Vacutainers tubes with heparin (5 ml), which were centrifuged within 1 h of collection for 15 min at 2500 g. These samples were also used in the other experiments described below.

Effect of clomiphene citrate on ROS plasma production

Blood samples (1 × 10⁵ cells) were suspended in 5.0 mmol/l phosphate-buffered saline (200 µl) both with and without clomiphene citrate for 60 min. Intracellular ROS production was detected in human leukocytes using the non-fluorescent cell-permeating compound 2,7′-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is hydrolysed by intracellular esterases to DCFH, which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by cellular oxidants. After clomiphene citrate exposure, the cells were treated with DCFH-DA (10 µmol/l) for 60 min at 37°C. The fluorescence was measured at an excitation of 485 nm and an emission of 520 nm. The calibration curve was performed with standard DCF (0–1 mmol) and the level of ROS production was calculated as nmol DCF formed/mg protein (Wallace et al., 2006).

Cytotoxicity of Val16Ala SOD2 PBMC after clomiphene citrate treatment

The effect of clomiphene citrate on PBMC viability was measured using a protocol similar to the PBMC culture assay described previously by Montagner et al. (2010). Briefly, the blood samples were collected and centrifuged for 15 min at 2500 g and then the cells were transferred to culture media containing 5 ml RPMI 1640 with 10% fetal calf serum and 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were cultured in a 96-well plate at a density of 1 × 10⁵ cells in 100 µl culture medium for 72 h at 37°C in a humidified 5% CO₂ atmosphere. The cells were counted, centrifuged for 10 min at 2000 g and transferred to a new culture media containing clomiphene citrate. The PBMC (1 × 10⁵ cells) were treated with clomiphene citrate for 24 h; each treatment concentration was performed in triplicate. After this period, PBMC viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann, 1983).

Statistics

All analyses were carried out using the Statistical Package for Social Sciences version 18.0 (SPSS, Chicago, IL, USA). The mean values were compared among different clomiphene citrate concentrations, using ascorbic acid as an antioxidant control compound, were determined using a one-way analysis of variance followed by a post-hoc Tukey’s test. The cytotoxicity effect of clomiphene citrate on PBMC from different Val16Ala SOD2 donor samples was compared using the two-way analysis of variance followed by a post-hoc Tukey’s test. All P-values were two-tailed. The alpha value was set to <0.05 to determine statistical relevance.

Results

Before evaluating the in-vitro effect of clomiphene citrate treatment on blood and PBMC with different Val16Ala SOD2 genotypes, the antioxidant capacity of clomiphene citrate was analysed using a DPPH assay and compared with that of the antioxidant molecule ascorbic acid at the same concentrations; these results are presented in Figure 1. The clomiphene citrate treatment caused some antioxidant...
effects. However, its antioxidant capacity was 24.5% of that of ascorbic acid (20 μmol/l).

The values of 50% scavenging activity (SC50) were also calculated from a regression curve. The concentrations required to scavenge 50% of DPPH radical was 35.764 μmol/l clomiphene citrate and 7.361 μmol/l ascorbic acid. Ascorbic acid was 4.85 times stronger than clomiphene citrate.

Clomiphene citrate treatment of in-vitro biological samples showed antioxidant effects on the blood total ROS production when compared with the control group without treatment (Table 1). However, the effect was not dose-dependent because a U-shaped curve was observed when analysing the effect of the clomiphene citrate concentrations. The highest antioxidant effect was observed with 5 μmol/l clomiphene citrate, while treatment with 20 μmol/l clomiphene citrate showed similar ROS production to that of the control group.

Additionally, variation in ROS production in response to clomiphene citrate treatment was noticed when the Val16Ala polymorphism genotypes were considered (Figure 2). The AA genotype was more responsive to clomiphene citrate treatments at 1, 5 and 10 μmol/l, as displayed by the significant decrease in ROS production that was observed when compared with the control group (P = 0.002). However, the blood from VV patients displayed an antioxidant effect on ROS production exclusively with 1 μmol/l clomiphene citrate. Heterozygous (AV) samples did not show any antioxidant effects following clomiphene citrate treatment when compared with the untreated group.

A third experiment was performed to assess the effect of clomiphene citrate on PBMC cytotoxicity. In general, clomiphene citrate treatments at concentrations of 10 and 20 μmol/l caused an increase in mitochondrial function, which indicated a positive influence on cell viability by clomiphene citrate. However, these results were also Val16Ala SOD2 dependent (Table 2).

The AA and AV PBMC samples showed an increase in viability with the 10 and 20 μmol/l clomiphene citrate treatments when compared with controls and low clomiphene citrate treatments. In these cells, the 20-μmol/l clomiphene citrate treatment resulted in higher viability than other treatments. However, these results were not observed in VV PBMC samples. In this genotype group, only the 5 and 10 μmol/l clomiphene citrate concentrations had an effect on cell viability.

**Discussion**

This work observed that clomiphene citrate displays an antioxidant capacity and an effect on oxidative metabolism as evaluated by ROS production. Additionally, clomiphene citrate treatments at some concentrations (predominantly 5 and 10 μmol/l) led to increased PBMC viability. However, perhaps the most important finding in this study was the potential pharmacogenetic effect of the Val16Ala SOD2 polymorphism on the variables analysed here. Although this study involved in-vitro experiments and was limited to a few samples with different Val16Ala SOD2 genotypes, these results are interesting and should be investigated further.

For the past four decades, clomiphene citrate, a SERM drug, has been used as the primary treatment for infertility, as recommended by the World Health Organization, for group II anovulatory patients (Duwe et al., 2010). In some cases, SERM may antagonize the effects of oestrogen by...
preventing oestrogen molecules from binding to receptor sites, while in other cases; these drugs exert oestrogenic activity by mimicking oestrogen. Additional investigations showed that SERM, such as tamoxifen and raloxifen, which are used in breast cancer and osteoporosis treatments, also exert antioxidant effects and, therefore, have positive biological effects on cardiovascular and neural physiology (Custo´dio et al., 1994; Herna´ndez-Esquivel et al., 2011; Morota et al., 2009; Ozbasar et al., 2010; Wong et al., 2008). However, as far as is known, previous studies of the antioxidant effects of clomiphene citrate were mostly preliminary. The results described here suggest that this SERM also exerts antioxidant effects.

However, the use of only two in-vitro protocols to test the clomiphene citrate antioxidant capacity (DPPH and DCFH) could to be considered limited since clomiphene citrate appears to be a less effective antioxidant when compared with ascorbic acid which shows at least 4-fold more antioxidant activity than clomiphene citrate (Figure 1). Although clomiphene citrate presents a lower antioxidant capacity when compared with ascorbic acid, clomiphene citrate appears to have some biological effect, as shown by the additional test that found an effect of clomiphene citrate on PBMC viability. Additionally, previous studies involving other SERM such as tamoxifen have suggested that these molecules present antioxidant properties. Custo´dio et al. (1994) described radical scavenging activity for tamoxifen and hydrotamoxifen peroxyl. However, Gerh´auser et al. (2003) tested a series of known chemopreventive substances including tamoxifen using several tests including the DPPH assay and found that for tamoxifen the half-maximal scavenging concentration was >250 µmol/l.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MTT reduction (MTT formazan/µg cell protein)</th>
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<tbody>
<tr>
<td></td>
<td>AA</td>
</tr>
<tr>
<td>Control</td>
<td>0.22 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Clomiphene citrate (µmol/l)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.19 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>0.27 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>0.51 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>0.60 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Different letters within each genotype group indicate statistical differences compared by one-way ANOVA followed by Tukey test (P = 0.04). Cytotoxicity is inversely related to MTT reduction.
whereas for ascorbic acid it was 8.5 μmol/l. Therefore, tamoxifen seems to have an antioxidant capacity even lower than the clomiphene citrate when compared with ascorbic acid.

Despite the lower tamoxifen antioxidant capacity, several studies describing its antioxidant properties have been published in the literature. We can cite the investigation performed by Cardoso et al. (2004) that observed protection of tamoxifen against oxidation of mitochondrial thiols and NADPH underlying the permeability transition induced by pro-oxidants and the study performed by Gharehbaghni et al. (2010). These authors described the effects of tamoxifen on lipid peroxidation and oxidant—antioxidant balance in an animal model. However, additional in-vitro and animal experimental model studies need to be performed to evaluate the potential causal mechanism of clomiphene citrate antioxidant activity. A clinical trial to test if clomiphene citrate administration has some influence on blood oxidative metabolism biomarkers could also help to clarify its antioxidant effect.

This study also observed that the clomiphene citrate antioxidant effects are influenced by the presence of the Val16Ala SOD2 polymorphism. These data are interesting because another independent research group recently described the association of this polymorphism with the success of IVF (Ruiz-Sanz et al., 2011). The authors studied 148 infertile women and found that the AA females showed a higher pregnancy rate when treated with IVF. Based on these results, the authors suggested that antioxidant defence, particularly in the mitochondria, could contribute to conception IVF success.

If SOD2 is indeed a key molecule involved in the female reproductive metabolicism, it can be speculated that alterations in SOD2 activity caused by the Val16Ala polymorphism could result in differential responses to drugs such as clomiphene citrate. However, whether its activity could have a pharmacogenetic effect on patients with clomiphene citrate resistance and pregnancy failure (Badawy and Elnashar, 2011). The reason that the pregnancies fail despite the increase in approximately 70% of women treated with clomiphene citrate, fewer than half become pregnant (Homburg, 2005). The reason that the pregnancies fail despite the increase in the ovulation induced by clomiphene citrate is not well understood. One cause of pregnancy failure is the oestrogen antagonist effects of clomiphene citrate in the cervical mucosa and endometrial tissue (Homburg et al., 1988).

Therefore, considering the results described by Ruiz-Sanz et al. (2011) and the observations reported in this study, the possible role of oxidative stress in these two scenarios (clomiphene citrate resistance and pregnancy failure) cannot be ignored. Although the present data were obtained from young healthy women without a history of infertility, it is important to point out that the previous studies performed by this research team described a link between the lower SOD2 activity associated with the V allele and obesity (Montano et al., 2009), hypercholesterolaemia and higher oxidative stress biomarkers (Duarte et al., 2010). As PCOS is closely associated with obesity and other metabolic disorders, these results indicate the potential effect of Val16Ala SOD2 polymorphism in PCOS. However, at the moment, association studies between PCOS and the Val16Ala SOD2 polymorphism cannot be located in the literature. The absence of clinical data could be considered the main methodological limitation of this study.

Another important methodological concern is related to the viability test. The reduction of MTT observed in the in-vitro assay is not exclusively for mitochondria, where SOD2 is found, since MTT can be reduced to formazan by many other NAD(P)H-reductases found in the cytosol and plasma membrane. Although the MTT assay is not specifically a mitochondrial superoxide indicator, this assay was used since the oxidative stress caused by gene polymorphism is apparently not exclusive to the mitochondria (Montagner et al., 2010). This is because the Val16Ala SOD2 polymorphism leads to oxidative unbalance that probably involves the modulation of several ROS molecules.

The VV genotype presents superoxide ion excess that may result from lower SOD2 efficiency and this molecule can react with NO present in the cells, producing peroxynitrite. Peroxynitrite is known to oxidize sulphhydrils and yields products indicative of hydroxyl radical reactions with deoxyribose and dimethyl sulphoxide. Thus, peroxynitrite may damage cells by promoting membrane lipid peroxidation and nitration of proteins on tyrosine residues (Rubbo et al., 1994). The production of lipid peroxides interferes with the regulation of several metabolic pathways as well as causing cellular membrane frailty. On the other hand, the conversion rate of superoxide to H2O2 associated with the A allele is more effective, but excessive superoxide dismutation to H2O2, which is an important oxidative molecule, can lead to the reaction of H2O2 with metal ions such as Fe2+ present in the cytosol and produce hydroxyl radicals. Therefore, the interaction of the clomiphene citrate antioxidant effect and Val16Ala-SOD2 polymorphism was investigated by evaluating PBMC viability with the MTT viability assay. However, the evaluation of the causal mechanism of the clomiphene citrate pharmacogenetic effect needs to be clarified in additional investigations.
the results observed in different genotypes are not directly influenced by ethnic background of subjects.
However, it was important to perform the initial in-vitro analysis described here, which contributes to the evaluation of the impact of the SOD2 polymorphism on the treatment of women with clomiphene citrate in a clinical setting. Complementary investigations must be performed that consider the potential association between the Val16Ala SOD2 polymorphism and PCOS, the oxidative status of infertile women before and during clomiphene citrate treatment and the potential pharmacogenetic influence of the Val16Ala SOD2 polymorphism.

The results described here shed light on several complementary studies that could help to understand the mechanisms involved in female infertility and may assist with the development of personalized treatments.

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