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Original Research Article

Evaluation of vitamin E levels, antioxidant enzymes and malondialdehyde in women with unexplained infertility

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ABSTRACT

Background: Unexplained infertility is a perplexing disorder. 30% of infertile couples are diagnosed with unexplained infertility with all standard fertility investigations normal. Oxidative stress has been shown to have a negative impact on reproductive function in women with endometriosis and (PCOS) polycystic ovarian syndrome. In another study antioxidant supplementation improved OS induced infertility. Many studies have demonstrated an increase in Malondialdehyde in serum of women with infertility and vitamin E as a protective antioxidant in the body with positive effect on the fertility. There is growing evidence linking OS and unexplained infertility.

Based on this knowledge the specific group of unexplained infertile women were chosen to assess their levels of serum antioxidant enzymes, Vitamin E and lipid peroxidation marker.

Aim: To compare the Levels of Malondialdehyde, Antioxidant enzymes and Vitamin E in the serum of women with unexplained infertility and control group.

Method: Case control study.

70 normal ovulatory women who conceived within 12 months of contraceptive free intercourse, and with no history of miscarriage were recruited in the control group. 70 women with unexplained infertility were recruited as study group. All participants included in the study were between 28 and 38 years of age. Serum levels of MDA, Antioxidant enzymes (GST, SOD, Catalase) and Vitamin E concentrations were compared between two groups. Vitamin E concentrations were determined by using High performance liquid chromatography. Antioxidant enzymes and MDA were measured by standard spectrophotometric assay.

Results: Data was analysed using SPSS Software. Continuous parameters were analysed using Mann Whitney U test. There is a significant decrease in the vitamin E levels 3.80 (ug/ml) in the unexplained infertile group as compared to controls 6.0 (ug/ml). All women in the study group showed significantly ($P<0.05$) higher levels of MDA and lower levels of antioxidant enzymes such as GST, SOD and Catalase as compared to the fertile women. This decrease was found to be significant with p value of <0.05.

Conclusion: The study group have a higher oxidative stress status and low level of antioxidants compared to control group. Serial measurement of oxidative stress biomarkers and their defense system may help to understand the aetiology of unexplained infertility and to enhance their chances of conception.

1. Introduction

Infertility is a multifactorial disorder and life style has an important role in its occurrence. In WHO conducted Demographic Health Surveys from 1990 to 2010, responses from women were evaluated and it revealed one in every

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four couples in developing countries were affected by infertility. It also reported that the overall burden of infertility, in women from 190 countries was similar and that increasing trend was seen in that decade.[1] This clearly shows that the burden remains high. According to Crosignani and Esteves, the evaluation of unexplained infertility in 30-50% of Couples was based on the following simple criteria:

1. Normal ovulatory function
2. Normal semen analysis for men
3. At least one patent fallopian tube[2]

Studies have shown that Assisted Reproductive techniques such as intrauterine insemination (IUI), In-vitro fertilization (IVF) are not always successful. It is worthwhile to investigate the factors which affect the success of this procedure.[3] Ovary is responsible for production of reproductive hormones and oocytes. The oxidative stress in reproductive organs may play a vital role in preventing conception. K. H. Al-Gubory et al. reported that levels of reactive oxygen species and antioxidants influences the female reproductive function in different phases of menstrual cycle and the physiological process of pregnancy.[4] Wang et al. (1997) and Polak et al. (2001) reported that higher levels of reactive oxygen species were found in peritoneal fluid of women with idiopathic infertility than fertile control. It was suggested that peritoneal fluid which enters the Fallopian tubes may cause damage to sperm, and creates oxidative stress (Storey, 1997). Fridovich in his study has shown antioxidant enzymes play critical roles in clearing the toxic products produced by superoxide dismutase.[5]

1.1. Role of vitamin E in fertility

Vitamin E the fat-soluble vitamin is found in the ovary especially in follicular fluid. Many studies have shown its role as an antioxidant within the body.[6] In cells and organelles vitamin E is the first line of defence against lipid peroxidation. Rigotti, A has shown role of vitamin E in RBC flexibility and longevity in immune function, and its positive effects on fertility.[7] Savita, et al. have shown that increased OS is associated with decrease in antioxidants and fertility.[8] Vitamin E directly neutralizes superoxide anion, hydrogen peroxide, and hydroxyl radical. It is called a chain breaking antioxidant because of its ability to terminate a free radical chain reaction. Ruder, E.H. et al have shown that Vitamin E increases the number of embryos developing into the expanded blastocysts and increases the viability of embryos exposed to heat shock.[9]

Bayer, R. (1960) suggested that in a human trial, infertile couples given vitamin E have shown a significant increase in fertility.[10] Plasma vitamin E levels were found to be higher in fertile women than in infertile women.[11] Vitamin E supplementation in older mice partially prevented reduction in ovulation[12] Vitamin E has a significant role in reproduction and sufficient levels give a better effectiveness in the treatment of unexplained infertile women.

1.2. Antioxidant status in females with unexplained infertility

Oxidative stress is due to the imbalance between pro-oxidant molecules and protective antioxidants. OS affects the entire reproductive capacity of a woman in many ways (i.e., oocyte maturation, ovulation, implantation, formation of blastocyst, luteolysis and luteal maintenance in pregnancy).[13] Reactive oxygen species act, both as key signalling molecules in pregnancy and at higher levels cause adverse effect on female reproductive tract. The antioxidants scavenge free radicals, thus protecting the cell structures.[14] Excess ROS in the follicular fluid overpowers the antioxidant defence capacity and damages the oocytes. This will affect the fertilization process. Peritoneal cavity microenvironment when flooded with ROS may allow fertilization but OS-induced apoptosis can cause implantation failure.[15] Elevated ROS levels hinder the endometrial function, which is needed for the growth of the embryo[16] and interferes in luteal regression and hormonal support which is responsible for the continuation of a pregnancy.[17]

1.3. MDA

There are studies showing increased marker of lipid peroxidation (MDA) in women with unexplained infertility when compared to primary infertile woman and male infertility. Chao HT et al and Tsuboi H etal showed that the oxidative damage could be due to factors like repeated drug induced ovarian stimulation or psychological stress.[18] This necessitates further evaluation of factors responsible for oxidative stress in infertility workup protocol. Majid KH etal clearly correlate primary female infertility with significantly high levels of MDA and decreased levels of antioxidants.[19]

1.4. Antioxidant enzymes

Gonadotropin accelerate the up regulation of antioxidants such as catalase in the follicles and protects oocytes from ROS during steroidogenesis.[20] Strong positive correlation was observed between SOD activity and intrafollicular oestradiol levels, which affects oocyte quality. Studies concluded that the ROS scavenging ability of antioxidant enzymes is related to fertilization outcomes[21] GST is involved in the follicle maturation process by detoxifying harmful substances and enhancing the normal development of the oocyte.[22] This study aims to establish the association of Oxidative stress, MDA and antioxidant defence in unexplained infertility.

2. Material and Methods

2.1. Study design

Case - Control study.

2.2. Control group

Pregnant women with normal ovulation who attend the antenatal clinic in Obstetrics unit. Samples were collected from them at first-trimester of their pregnancy. 70 of these participants who had an uneventful pregnancy were included as control group.

2.3. Inclusion criteria

Women with normal ovulation, aged between 28 and 38 years who have conceived within 12 months of contraceptive free intercourse, and who have an uneventful pregnancy.

2.4. Exclusion criteria

1. Abnormal glycaemic status and thyroid function (TSH and Hb A1c were measured).
2. History of adverse effect during pregnancy.

2.5. Cases

70 married Women with unexplained infertility were diagnosed in the Reproductive Medicine Unit at CMC, Vellore when they came for infertility treatment procedure.

2.6. Inclusion criteria

1. Women with unexplained infertility were aged between 28 and 38 years.
2. Women with normal results for the following tests.
   (a) Tubal patency (hysterosalpingogram and/or laparoscopy documents at least one fallopian tube patent.
   (b) Normal ovulatory function (Regular menstrual history /ultrasound documented ovulatory cycle or mid-luteal Progesterone).
   (c) Normal semen analysis for their partners.

2.7. Exclusion criteria

Women in whom one or more of the above test results is abnormal.

2.8. Sample size

In order to detect this difference as statistically significant with an alpha error of 5% and the power of 90%, we included 70 unexplained infertile women and 70 controls who were normal.

Study approved by IRB and consent was obtained from all participants.

2.9. Statistical analysis

Data was analysed using SPSS Software. Continuous parameters were analysed using Mann Whitney U test. P value of < 0.05 was considered significant.

2.9.1. Sample collection and preservation

Blood samples (5 ml) were collected in serum tubes (red clotted tube) and centrifuged at 3000 rpm for 10 min. Serum were separated and stored in 0.5ml micro tubes at -20°C until analysis.

2.9.2. Analytical methods

Vitamin E- was quantified using High performance liquid chromatography (HPLC).

2.9.3. Chromatographic conditions

1. Stationary phase - Phenomenex analytical column (C18 column, 5mm particle size, 250x4.6mm).
2. Mobile phase - Methanol. Detector- (PDA) photo diode array.
3. Vitamin E Retention Time =12.9 ± 0.092.

2.9.4. Calibration

Vitamin E Standard stock solution was prepared by dissolving 10mg of alpha-tocopherol in 1ml ethanol working standards 12.5, 25, 50 and100 ug/ml were prepared from stock 10mg/ml tocopherol acetate internal standard stock solution was prepared in ethanol.

2.9.5. Steps in sample preparation

1. 100 μl of serum with 100 μl internal standard were mixed for 15 sec in a vortex mixer.
2. Tube kept in ice for 5 min. 1 ml of hexane added and vortexed for exactly 1 min.
3. Centrifuged at 25°C for 5 min at 1,500 rpm.
4. Upper clear hexane layer was separated and evaporated under nitrogen gas.
5. 100μl of methanol was added and vortexed for 15 seconds.
6. The aliquot was transferred to a HPLC glass vial and loaded for further analysis.

The Figures 1, 2, 3 and 4 represents the peak of different concentration of vitamin E standards.

2.10. Limit of quantification: (LoQ)

1. The procedure was able to detect up to 3 μg/ml with accuracy.
2. The measurements were made on replicate samples (n=6) during the same laboratory run.
3. The values were within ± 2 SD of the mean.

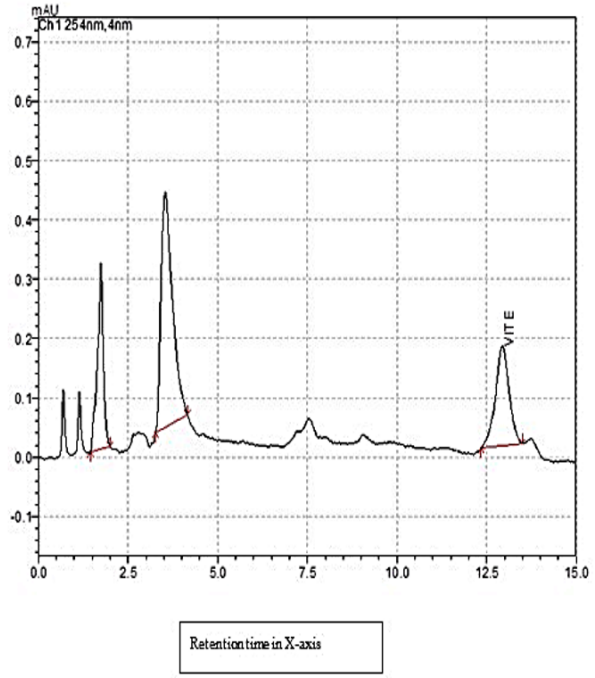

Fig. 1: Standard 12.5ug/ml

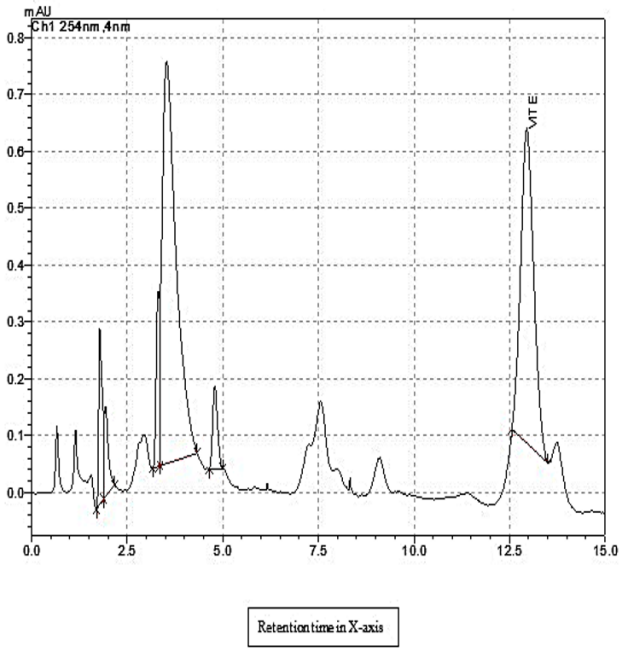

Fig. 2: Standard 25ug/ml

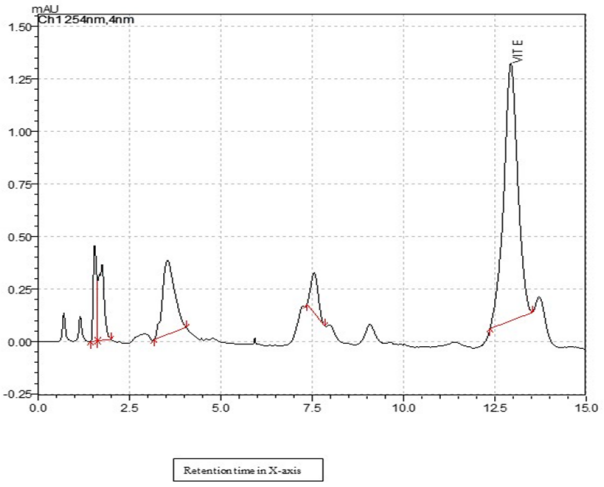

Fig. 3: Standard 50ug/ml

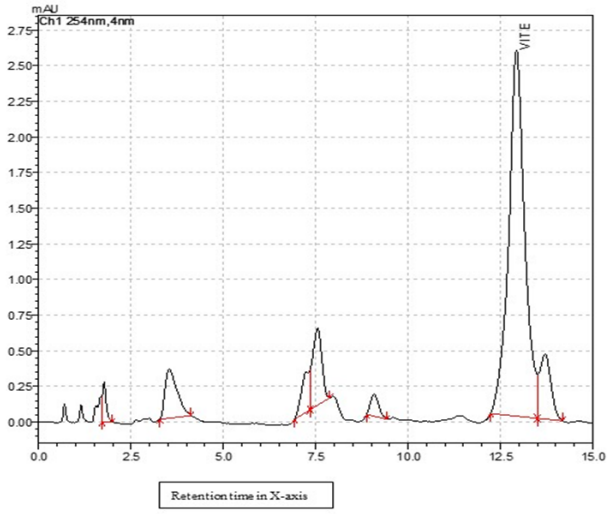

Fig. 4: Standard 100ug/ml

2.11. Antioxidant enzymes

1. **Catalase** – measured by Mahmoud H. Hadwan principle:
   2H2O2 Catalase → 2H2O + O2
   Catalase activity was measured by incubating the sample in 1.0 ml substrate at 37°C for 3 minutes. Ammonium molybdate was added to stop the reaction. Absorbance of the yellow complex of molybdate and hydrogen peroxide is measured at 374 nm against the blank.
2. **GST** – measured by Boyland, E. and Chasseaud using CDNB as a substrate principle
   Glutathione –SH + → Glutathione –S-CDNB
   The reaction is assessed by monitoring the conjugation of 1-chloro, 2, 4-dinitrobenzene (CDNB) with reduced

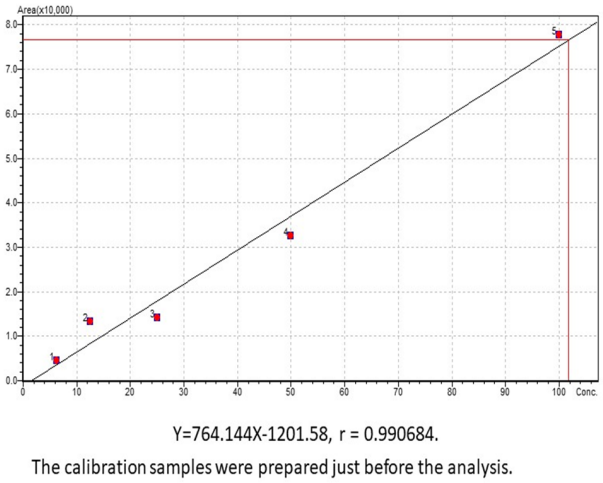

Fig. 5: Calibration graphof Vitamin E standards

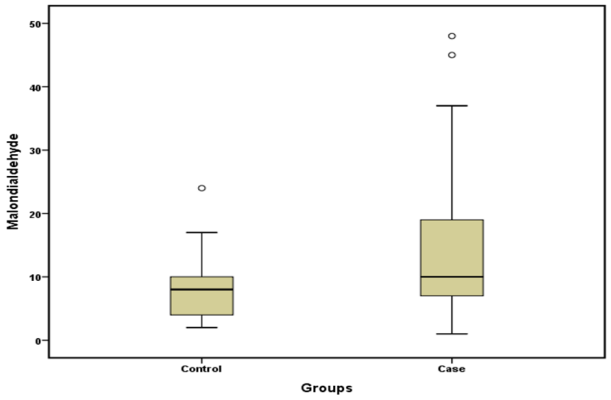

Fig. 6: Box plot for MDA (umol/L) Vs Case / Control

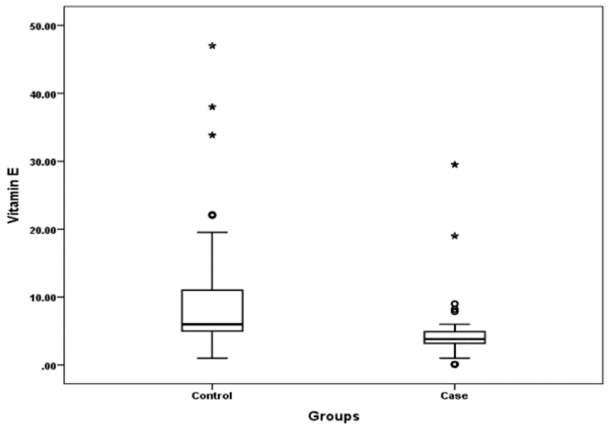

Fig. 7: Boxplot for serum vitamin E((ug/ml) between control and study group

glutathione (GSH). This is done by an increase in absorbance at 340nm.

3. **SOD** – measured by Markland's method measured by Markland's method
   Principle: Superoxide dismutase reacts with pyrogallol and inhibits its autooxidation.
   The rate of autooxidation of pyrogallol calculated from the absorbance of orange colour compound at 420nm.
4. Lipid peroxidation marker (MDA)- measured by Thiobarbituric acid method
   Principle: The lipid in the cell membranes, highly susceptible to oxidative damage is broken down in to an number of units to form Thiobarburicacid reactive substances (TBARS), with Thiobarbituricacid.

3. Results

A total of 140 women, (70 normal and 70 women with unexplained infertility) in the reproductive age group of 28-38 were included in this study. The mean ages of the participants were 26.70 ± 4.40 and 30.39 ± 4.36 respectively for control and cases. (Table 1)

The median of MDA level was found to be significantly higher in cases than in control ($p < 0.005$) (Table 3).

This is clearly represented in boxplot (Figure 7)

The antioxidant enzyme activities were significantly lower in case group compared to the control subjects (Table 3).

SOD showed significant decrease in median value in study group compared to control group. p=<0.001.

Catalase activity in study group decreased compared to control group p= <0.001.

GST in serum levels showed significant decrease in median compared to control group p =< 0.001.

Unexplained infertile group showed lower levels of vitamin E in serum compared to control group.

This difference were statistically significant. This is clearly represented in boxplot (Figure 7).

Serum vitamin E analysis by HPLC method showed good recovery.(Table 2)

4. Discussion

Unexplained infertility is a major problem with a significant public health concern. ROS and reactive nitrogen species (RNS) act as signal molecules in physiological and pathological process in female reproductive tract.[23] In this study the mean serum levels of MDA were significantly higher in the unexplained infertile group (Table 3), a finding similar to the study by Majid KH et al.[24] The higher MDA level in unexplained infertile group shows that there is considerable oxidative damage in this group of women decreasing the possibility of conception. This finding also implies the high levels of ROS has used

Table 1: Baseline characteristics of study subjects

Variables	Groups		P value
	Control(n=70) Mean ± SD / Median (IQR)	Case(n=70) Mean ± SD / Median (IQR)	
Age(yrs)	26.70 ± 4.40	30.39 ± 4.36	< 0.001
Body Mass Index(kg/m2)	23.14 ± 2.41	24.54 ± 4.27	0.02
HbA1C (%)	5.27 ± 0.43	5.28 ± 0.37	0.888
Height(cm)	155.55 ± 5.48	154.53 ± 6.66	0.323
Weight(kg)	55.89 ± 5.49	58.74 ± 10.56	0.049
TSH*	1.56 (1.11, 2.10)	2.08 (1.60, 2.85)	< 0.001

Note: * represents the variables which are reported by Median (IQR)

Table 2: Descriptive statistics for antioxidant enzyme, MDA and Vitamin E

Variables	Control Mean ± SD / Median (IQR)	Case Mean ± SD / Median (IQR)	P value
Superoxide Dismutase*(U/ml)	2.00 (2.00, 3.00)	1.00 (1.00, 2.00)	< 0.001
Catalase*(U/ml)	34.00 (27.00, 45.00)	23.50 (13.00, 35.00)	<0.001
GST Enzyme(U/ml)	1.87 ± 0.61	1.26 ± 0.53	< 0.001
Malondialdehyde* (umol/L)	8.00 (4.00, 10.00)	10.00 (7.00, 19.00)	0.001
Vitamin E* (ug/ml)	6.00 (5.00, 11.00)	3.80 (3.20, 4.90)	< 0.001

Note: * represents the variables reported by Median (IQR)

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Vitamin E* (ug/ml)	6.00 (5.00, 11.00)	3.80 (3.20, 4.90)	< 0.001

Note: * represents the variables reported by Median (IQR)

up the antioxidant enzymes in scavenging them. Studies have shown ROS over production in ovulatory follicles may affect the oocytes. Higher MDA concentration has been noted in human endometrium with negative impact on menstruation process.[25] Pregnant women were more susceptible to oxidative stress compared to non-pregnant women with increased ROS and decreased antioxidants.[26] So, evaluating the oxidative marker in the unexplained group would be helpful for their treatment.

Adequate levels of antioxidants are important for oocyte quality, maturation, fertilization, and implantation. They have a vital role in reducing oxidative stress, a process known to impair conception and its sustenance.[27] Various studies on Antioxidant enzymes in serum levels confirmed decreased levels of catalase, SOD and GST in association with infertility.[28] The results of this study clearly support this finding. Infertile couples were suggested antioxidants in their diet and also adopt a healthy life style.[29] Very few studies on oxidative stress markers like catalase, SOD, GST and MDA were studied collectively in Indian women with unexplained infertile woman. Findings from this study emphasizes the non-invasive protocol including oxidative stress markers.

Vitamin E (α-tocopherol) the lipid-soluble antioxidant is considered a "fertility factor". It is said to be a direct free radical scavenger by enhancing the antioxidant enzymes and protects the cell membranes from lipid peroxidation. In this present study, unexplained infertility group have lower concentration of serum vitamin E levels as compared with control group (p= <0.001). This study points out the importance of vitamin E and its effect on female fertility. Many studies showed low Vitamin E levels in infertile women with reduced total antioxidant status. According to Naseer et al Vitamin E levels in sera and cervical secretions of infertile women with unexplained infertility show a significant decrease when compared with fertile controls.[30]

5. Conclusion

The study clearly shows an imbalance in the antioxidant status with increased MDA level, decreased vitamin E and

antioxidant enzyme level in the study group as compared to control group. Vitamin E levels can be measured using this HPLC method and can be titrated to optimal levels. The antioxidant enzymes if included in the fertility workup and found deficient, can be given orally in the treatment protocol.

6. Limitation

Limitations of our study is the small number of subjects taken. A larger group might have been helpful in drawing better correlation between the various parameters. Other OS markers could have been included.

Interventional study with antioxidant supplementation and evaluation of OS markers before and after the treatment can be the future study. Measurement of these markers in follicular fluid can also be undertaken.

7. Source of Funding

This research project received funding from the IRB-Christian Medical College and Hospital, Vellore, with IRB reference number 9216, dating back to December 2014.

8. Conflict of Interest

None.

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