

The Utility of Plasma Transthyretin and Antioxidants as a Biomarker of Early Pregnancy Loss - A Pilot Study

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Abstract

Objective: Miscarriage or early pregnancy loss (EPL) affects about 15-25% of all pregnancies and is considered as one of the most common complications of the human reproduction. The objective of this pilot study was to quantify and compare the levels of plasma Transthyretin (TTR) and total antioxidant capacity (TAC) among EPL patients and normal pregnant women. **Design:** Cross sectional pilot study. **Subjects:** Patients diagnosed with EPL in their first trimester (Incomplete and Missed abortion) and medically terminated pregnancy (MTP) and pregnant women in their first trimester of pregnancy without any complications. **Methods:** Plasma TTR and total antioxidant capacity were quantified using Enzyme Linked Immunosorbent Assay (ELISA) and Trolox equivalent antioxidant capacity assay respectively. Statistical analyses were performed with SPSS software version 16.0 (SPSS Inc., Chicago, IL). All p values <0.05 were considered statistically significant. **Results:** A total of 60 subjects were enrolled in the study. There was no significant difference in the mean concentration of plasma TTR among women with EPL 322.20(187.33-444.10) µg/ml and normal pregnant women 224.32(176.06-509.97) µg/ml (p=0.501). However, a significant difference was observed in the TAC among women with EPL 0.58 (0.51-0.70) mM/µl and normal pregnant women 0.71 (0.63-0.79) mM/µl (p=0.006). No significant correlation was seen between TTR and antioxidant capacity. **Conclusion:** Our study failed to demonstrate any relationship between plasma TTR levels and EPL. Antioxidant capacity was lower among EPL patients suggesting the importance of supplementation with antioxidants to prevent poor outcomes in pregnancy.

Keywords: Early pregnancy loss, Transthyretin, Antioxidants

Introduction

Miscarriage or early pregnancy loss (EPL) affects about 15-25% of all pregnancies, and is considered as one of the most common complication of the human reproduction. The most common etiologies of EPL include the genetic or chromosomal abnormalities in the developing embryo, endocrine diseases, immunological factors, chemical agents, hereditary disorders, and psychological factors. Oxidative stress which is defined as an imbalance between pro-oxidants and antioxidant capacity has been implicated in many reproductive and pregnancy disorders such as sub fertility to miscarriage, maternal vascular disease and preterm labour [1].

Burton and Jauniaux et al. suggested that there was an increase in the oxidative stress markers in placenta tissue among EPL patients when compared to controls. This indicated an increase in the reactive oxygen species (ROS) which was due to premature establishment of maternal perfusion. Thus, the increase in oxidative stress may be associated with EPL [2].

Transthyretin (TTR), a 56-kDa homotetrameric protein, belongs to a group of proteins including thyroxine-binding globulin and albumin which is mainly synthesized in the liver, eye and choroid plexus. TTR is down regulated in the placental villous trophoblasts of early pregnancy loss in human. TTR plays a critical role in the transportation of the thyroid hormone, thyroxine (T₄) in the blood and retina through an association

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Original Research Article

with retinol binding protein (RBP); which are both critical for the neurological development of the fetus [3]. The secretion of TTR from the placenta is known to play a vital role in the transportation of the maternal thyroid hormone to the fetal circulation through the formation of the TTR-T₄ complex. The formation of this complex protein TTR-T₄ is considered important for the normal development of the fetus [4, 5].

It has been observed that the fetus begins to secrete thyroid hormone only after 12 weeks of gestation and until this time the fetal thyroid hormone supply originates from the mother and this is thought to continue throughout the pregnancy period.

The perseverance of high levels of deiodinase type 3 activity in the placenta is thought to inactivate T₄ to its inactive form rT₃. The existence of placental TTR in the 6th week of gestation will aid in the binding maternal T₄, which in turn will prevent deiodination and its delivery to fetal capillaries [6].

Down regulation of TTR during the first trimester pregnancy may lead to fetal growth restriction and development of pre-eclampsia [7-9]. Therefore, we hypothesized that the plasma levels of TTR might be low in EPL, suggesting that TTR might be used as a suitable diagnostic marker for EPL and a potential therapeutic target in preventing subsequent miscarriage.

The deficiencies in the antioxidant activities might result in poor pregnancy outcomes such as fetal growth restriction and preeclampsia [10]. Therefore, the measurement of plasma antioxidants can be a suitable diagnostic novel marker for EPL. Hence, we also attempted to measure the antioxidant concentration among study subjects. Therefore, the objective of our study was to quantify the levels of plasma TTR and total antioxidant capacity among patients with EPL and patients with normal pregnancy.

Methods

Study Population, type of study and ethical consideration: This was a cross-sectional pilot study performed in the Department of Obstetrics and Gynaecology and Clinical Pharmacology of a tertiary care hospital between January and August 2017. The study was approved by the institutional ethics committee. The study was Compliant with the Ethical Requirements. The study involved human participants.

The Informed Consent Informed consent was obtained from all individual participants included in this study.

Inclusion and exclusion criteria: Patients were diagnosed with EPL in their first trimester (Incomplete and Missed abortion) were considered eligible for inclusion into the study as cases. Patients who visited the outpatient department for medically terminated pregnancy (MTP) and pregnant women in their first trimester of pregnancy without any complications were considered eligible for inclusion as normal controls (NCs). We excluded patients with baseline hypertension, proteinuria, renal disease, diabetes, and twin pregnancy. Other exclusion criteria included patients with any major or minor fetal anomaly and chromosomal abnormalities.

Clinical collection of data: Clinical and demographic data were collected on patient age, waist and hip circumference, body mass index (BMI), gestational weeks and last menstrual period (LMP). Data were also collected on other risk factors such as consumption of coffee and use of oral contraceptives (OCPs). Blood sampling was done to determine the level of hemoglobin (Hb), serum creatinine (SCr), blood urea and platelet count.

Laboratory Investigations

Enzyme Linked Immunosorbent Assay (ELISA): After obtaining the written informed consent from all patients, 4 ml of blood sample was collected from the ante-cubital vein in the forearm. After adequate centrifugation at 2500 revolutions per minute (rpm) for 10 minutes, the plasma samples were extracted and were stored in -80°C deep freezer. The plasma which was centrifuged from the blood sample was subjected to competitive enzyme-linked immunosorbent assay (ELISA) for the measurement of protein TTR.

The assay was performed according to the manufacturer's instructions provided in the commercially available Human Transthyretin ELISA Kit (Bioassay Technology Laboratory, Cat. No E0000Hu, Shanghai, China). Inter and intra-assay coefficient of variation (CV's) was 6% and 8%, respectively. The measurement of TTR was done in a blinded fashion as the investigators who performed the ELISA assay were not informed about the patient's diagnosis.

Antioxidant assay: Plasma antioxidant capacity was measured by TEAC (Trolox equivalent antioxidant capacity) assay. It was performed according to the manufacturer's recommendations as provided in the commercially available kit (Antioxidant Assay kit, Sigma-Aldrich, Missouri, USA).

Original Research Article

The assay is based on scavenging of ABTS⁺ which is produced from oxidation of ABTS [2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)]. Antioxidants available in the plasma sample derived from patient's blood sample neutralize the radical cation, ABTS⁺ in a concentration dependent manner. Since ABTS⁺ is a chromogenic compound, the colour intensity was determined by spectrophotometer at 405 nm (Infinite F50 ELISA Plate Reader, Tecan Group Ltd, Mannedorf, Switzerland). It utilizes Trolox, a synthetic water-soluble antioxidant analogous to Vitamin E, which is used to express standard and samples as Trolox equivalent (mM) based on a Trolox standard curve. Inter and intra-assay CV's were 7% and 8%, respectively. The lab measurements were done in a blinded fashion as the investigators who performed the assay were unaware about the patient's diagnosis.

Statistical Analysis: The normality of data for continuous variables was checked using Q-Q plot in SPSS. Continuous variables were summarized as mean \pm standard deviation (SD) or median (IQR) and categorical data were expressed as frequency and percentages. Differences in the categorical variables between groups were evaluated using the chi-square test. Parametric or non-parametric tests were used based on the distribution of data. The differences in continuous variables between groups were analyzed with Mann-Whitney U test or Independent Sample t Test based on normality of data. Spearman and Pearson correlation was done to establish the relation between the laboratory assays. All statistical analyses were performed with SPSS software version 16.0 (SPSS Inc., Chicago, IL). All p values <0.05 were considered statistically significant.

Results

A total of 60 subjects were enrolled in the study. The study participants comprised of two groups: patients with missed/incomplete abortion (n=30) and controls comprised of patients with uncomplicated pregnancy (n=30). All information on the baseline demographic data, clinical variables, laboratory investigations and risk factors were collected (Table 1). There were no significant differences in the baseline demographic and clinical variables between the two groups. Significant differences were not observed in the laboratory investigations, platelet count, blood urea and serum creatinine between the two groups.

Table-1: Baseline Characteristics Including Demographics, Risk Factors and Laboratory findings of Study Patients

Patient Characteristics	Missed Abortion/Incomplete Abortion (n=30)	Uncomplicated/Normal Pregnancy (n=30)	p-value
Age, years	25.33 \pm 3.94	24.93 \pm 3.80	0.285
BMI, kg/m ²	22.91 \pm 4.73	24.85 \pm 3.68	0.186
Systolic Blood Pressure, mm Hg	111.72 \pm 5.31	110.41 \pm 9.40	0.808
Diastolic Blood Pressure, mm Hg	72.33 \pm 8.98	73.05 \pm 8.30	0.783
Heart Rate, PR	81.63 \pm 5.01	79.55 \pm 6.73	0.214
No. of gestational weeks	9.56 \pm 3.02	8.96 \pm 3.49	0.508
Platelet count, per cu mm	282840 \pm 53434.6	228760 \pm 97411.2	0.067
Blood urea, mg/dL	15.28 \pm 5.76	15.50 \pm 3.16	0.919
Serum creatinine, mg/dL	0.49 \pm 0.12	0.5 \pm 0.05	0.780
Coffee Intake (Yes/No)	2(6.7%)	7(23.3%)	0.070
Use of Oral Contraceptives (Yes/No)	2(6.7%)	3(10%)	0.316

There was no significant difference in the median concentration of plasma TTR among patients with missed/incomplete abortion and controls [322.20 (187.33-444.10) μ g/ml versus 224.32 (176.06-509.97) μ g/ml (p=0.501)] respectively (Figure 1a). However, a significant difference was observed in the median total antioxidant capacity among patients with missed/incomplete abortion and controls [0.58 (0.51-0.70) mM/ μ l versus 0.71 (0.63-0.79) mM/ μ l (p=0.006)] respectively (Figure 1b). No significant correlation was observed between plasma TTR and antioxidant capacity. Furthermore, no significant correlation was observed between gestational weeks, and antioxidant capacity and gestational weeks respectively.

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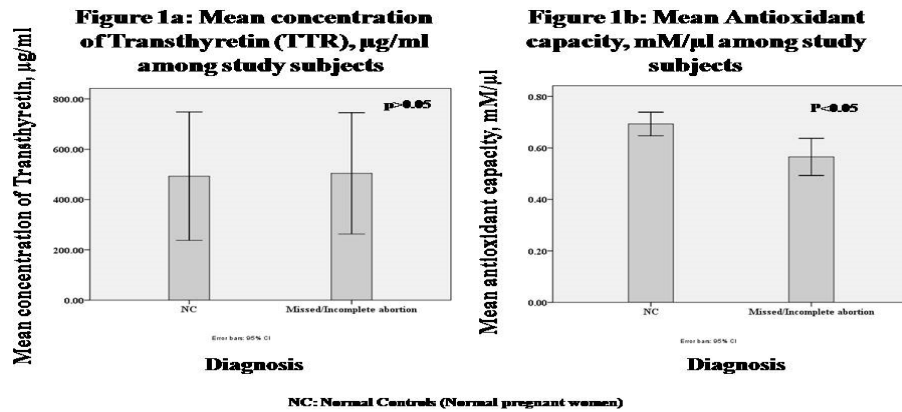


Figure 1a: A bar diagram demonstrating the differences in the mean concentration of Transferrin, µg/ml among study patients with early pregnancy loss (EPL) and normal pregnant (NC) women.

Figure 1b: A bar diagram demonstrating the differences in the mean antioxidant capacity, mM/µl among study patients with early pregnancy loss (EPL) and normal pregnant (NC) women.

Discussion

To the best of our knowledge this is the first clinical study that studied and compared the plasma levels of TTR among women with EPL and uncomplicated pregnancy. The present study compared the levels of TTR among women with EPL and uncomplicated pregnancy.

There are only few clinical studies that measured the plasma levels of TTR among pregnant women in their first trimester. However, most of these studies were performed among pregnant women with preeclampsia [9, 11-12]. The TTR levels observed in our study (224.32 µg/ml) were in consensus with the study performed by Fruscalzo et al (211 µg/ml) [9].

The present study failed to demonstrate any significant difference in the plasma levels of TTR among women with EPL and women without any complications in their pregnancies. Our study results did not match the results of the study which was performed by Liu et al. and colleagues, where proteomic analysis on the alteration of protein expression in the placental villous tissue of EPL was done. The study revealed that there were reasonable decreases in the grade of TTR protein staining in the cytoplasm of syncytiotrophoblastic and/or cytotrophoblastic cells in placental villous tissues obtained from the EPL group compared to women with uncomplicated pregnancy [13].

Although in a study performed by Episkopou et al. TTR deficient mice reported normal fertility, some of the recent studies reported that the protein might play an important role in the early stages of pregnancy [14]. The down regulation of TTR, a protein involved in the vitamin A and thyroid hormone metabolism may play a vital role in the pathological development of EPL.

However, studies have reported that the progesterone receptor plays a role in the up regulation and synthesis of TTR, secretion and uptake of TTR by human placenta have also been reported. Recent studies have reported the internalization of TTR by trophoblast and in the presence of T4, there is an increase in the uptake of TTR [4, 15-16]. Therefore, in consensus with our study it may be hypothesized that an increase in the plasma levels of TTR among women with EPL than in women with uncomplicated pregnancy can be explained by the theory of decreased internalization of TTR by trophoblasts.

In EPL there is deficiency of progesterone as well as deficiency of T4 in the maternal circulation which may prevent the internalization of TTR and thus increase the serum level of TTR and simultaneously decrease the local TTR at placental villous tissue [17,18].

Furthermore, clinical studies measuring TTR levels were performed among pregnant women with preeclampsia. The studies showed that TTR levels were lower among women with severe preeclampsia in each month of gestation than among women with normal pregnancy [9, 11-12]. Therefore, the production of TTR by trophoblasts demonstrated a mechanism that helps in the transfer of maternal thyroid hormone to the fetal circulation which could have clinical implications in the fetal development [6]. Our study showed that the total antioxidant capacity was significantly higher among women with uncomplicated pregnancy when compared to women with EPL. Similar results were seen in other studies where the antioxidant capacity was lower among women who had a first trimester miscarriage than that of healthy women in their first trimester pregnancy [19].

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The total antioxidant status (TAS) and copper (Cu) was significantly lower among women who experienced EPL than that of the normal pregnant women. In another study performed by Jenkins et al., it was observed that women suffering from EPL had significantly lower levels of antioxidants superoxide dismutase (SOD) when compared to women whose pregnancies were successful [20]. During the first trimester pregnancy, the abnormal placentation would cause oxidative stress that result in the endothelial dysfunction. This would lead to the emergence of pregnancy complications such as abortion [21]. The excess production of reactive oxygen species (ROS) will lead to the cellular damage of DNA, lipids and cellular proteins.

The supplementation of antioxidants can protect the cells from peroxidation reactions that can prevent cellular damage and can also help in the maintenance of cellular integrity [22].

Furthermore, studies have also shown that oxidative stress is associated with recurrent pregnancy losses [23-25]. Therefore, measuring the antioxidant levels might be considered as a novel diagnostic marker for EPL and supplementation with adequate antioxidants might provide a substantial therapeutic benefit. We also attempted to study the relationship between TTR and gestational weeks among the study patients.

However, our study failed to produce any correlation between gestational age and TTR. In a study, the changes in placental TTR in early and late pregnancy was measured where placentas were collected from the surgically terminated pregnancies between 6 and 17 weeks of gestation and compared with the normal term between 38 to 39 weeks following the caesarean section. The laboratory methods used for deterring the TTR mRNA and protein levels were Real time-Polymerase Chain Reaction, western blotting and immunohistochemistry. The study demonstrated a significant increase in the expression of TTR mRNA and TTR proteins between 6th and 13th weeks of gestation; however, this increase in expression was not seen between 13th and 17th weeks [26]. Our study being limited to women in their first trimester of pregnancy (<12 weeks) may be one of the reasons for the failure to produce any significant correlation between TTR and gestational weeks. During the first trimester of pregnancy the placenta remains relatively hypoxic and there will be an increase in the oxygen level to an average of about 2.5% at 8 weeks and 8.5% at 12 weeks which remains stable until delivery [27, 28].

The increase in the levels of oxygen leads to the production of TTR in JEG-3 human choriocarcinoma cells. Therefore, it has been hypothesized that the production of TTR is increased during the first trimester pregnancy and the expression remains constant throughout the gestation [29]. Our study is a preliminary, pilot study with a small sample size and the samples being collected at different points of time during first trimester. Due to logistic reasons, we did not measure the mRNA expression of TTR among the study patients from the products of conception, an indirect method of estimation of TTR locally at placental tissue.

Our study failed to demonstrate any relationship between plasma TTR levels and early pregnancy loss. Antioxidant capacity was lower among EPL patients suggesting the importance of supplementation with antioxidants to prevent poor outcomes in pregnancy. Future studies should be directed towards understanding the role of transthyretin in early pregnancy loss using a larger cohort of subjects.

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Conflict of interest: The authors declare that they do not have any conflict of interest. All authors have read and approved the manuscript.

Author contributions

1. Maitrayee Senwas involved in the conception and design of the work, analysis and interpretation of data for the work, drafting of the manuscript and approving the final version to be published.
2. Luxitaa Goenka was involved in the acquisition of data for the work, analysis and interpretation of data for the work, drafting of the manuscript and approving the final version to be published.
3. Durga Jha was involved in the acquisition of data for the work, analysis and interpretation of data for the work, and approving the final version to be published.
4. Vijay Raj was involved in the acquisition of data for the lab work, analysis and interpretation of data for the work, and approving the final version to be published.
5. Soniya Charles was involved in the acquisition of data for the lab work analysis and interpretation of data for the work, and approving the final version to be published.

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6. Kanchana Mala was involved in the acquisition of data for the lab work analysis and interpretation of data for the work, and approving the final version to be published.

7. Melvin George was involved in the conception and design of the work, analysis and interpretation of data for the work, drafting of the manuscript and approving the final version to be published. Melvin George is also accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Impact Statement**What is already known on this subject?-**

Transthyretin has been associated with the neurological development of fetus and it is down regulated in the placental villous trophoblasts of early pregnancy loss in human. Oxidative stress has been associated with many reproductive complications such as infertility, miscarriage, pre-eclampsia, fetal growth restriction and preterm labour.

What the results of this study add?- The present study is in consensus with the existing literature that measurement of total antioxidant capacity could be a suitable diagnostic marker for EPL; however, the present study failed to demonstrate relationship between plasma Transthyretin and early pregnancy loss.

What the implications are of these findings for clinical practice and/or further research?

The supplementation of antioxidants can prevent poor pregnancy outcomes. Future studies should be directed towards understanding the role of transthyretin in early pregnancy loss using a larger cohort of subjects.

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Permission from IRB: Yes

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