Research Papers

Salivary HSP70 and Progesterone are Upregulated in Preeclamptic Antenatal Mothers

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ABSTRACT

Introduction and Aim: Most of the pregnancies are complicated with complexities due to the environment, psychological, physiological circumstances. It is crucial to diagnose the pregnant women for complications during the earlier stages of pregnancy. Most importantly, the ability to monitor health status, disease onset, and the progression and treatment outcome through non-invasive means is a highly desirable goal in healthcare management. In that, the saliva represents a suitable, potential, and alternative biological filtrate/diagnostic fluid/medium for exploring the surveillance of health and disease. Also, it offers further opportunities as a pool containing a vast repertoire of specific, biologically-active peptides and proteins. Since hypertension represents the major risk contributing to diverse pregnancy complications; the present study is aimed to quantify the level of HSP70 and Progesterone in the saliva of normotensive and preeclamptic antenatal mothers.

Materials and Methods: A saliva sample was collected from normotensive and preeclamptic antenatal mothers (n=10 each group) by passive drool method. Progesterone and HSP70 were quantified using ELISA kits.

Results: The present study shows an increase in the expression of HSP70 and Progesterone in the salivary sample of preeclamptic pregnant women.

Conclusion: Results with a higher value of HSP70 protein expression suggests that salivary HSP70 may serve as a novel and valuable marker in diagnosing pregnancy complicated with hypertensive disorders and salivary progesterone may be used as an indicator for imminent delivery in pregnant women.

Key Words: Biological filtrate/diagnostic fluid, Heat shock protein 70 (HSP70), Passive drool method, Preeclampsia, Progesterone, Saliva sample

INTRODUCTION

Heat shock proteins (HSPs) are traditionally recorded as intracellular chaperone proteins with multiple defense activities. Recent data indicate that HSPs are also found in the extracellular space where they may signal via membrane receptors to alter gene transcription and cellular function. Therefore, there is an increasing interest in estimating the expression of HSPs in the extracellular fluids and their correlation with existing clinical disorders. Our particular interest is the evaluation of HSP70 (M.W: 70kDa) in the saliva of normal and complicated pregnant women to elucidate its role in modulating immunodefense and associated protective function in pregnant women.

HSP70 proteins are ubiquitous molecular chaperones that function in a myriad of biological processes. At cellular premises, they modulate protein folding, degradation, transport across membranes, and also protein-protein interaction (18). These molecular chaperones were considered to be intracellular until recently they were also found extracellularly in human blood sera, in pancreatic juice, etc. (13,24). Fabian et al. 2003 reported the presence of HSP70 in human saliva, indicating its effect on the mucosal surface. HSP70 is also known for its cytoprotection

outside the cell through modulation of cytokine release and immunity (either immunostimulatory or immunosuppressive depending upon the cellular condition) (25). The cytoprotective properties may occur through cell-surface association. Since Saliva covers all the surface of the oral cavity, the function of salivary HSP70 may become more pronounced; where the molecules responsible for the cellular events appear on the surface of the oral cavity.

The salivary HSP70 may originate from sources like salivary glands, mucosal cells, the periodontal tissues, etc. About 65% of un-stimulated (resting) saliva originates from the submandibular gland, 25% from the parotid, 4% from the sublingual and 8% from other salivary glands. These percentages vary under stimulation, principally for an increased contribution of parotid saliva > 50%, submandibular ~ 35%, sublingual and the minor mucous glands ~ 7-8% each (4,10,2). Human saliva is composed of 98% water, while the other 2% consists of other compounds such as mucus, electrolytes, antibacterial compounds, and various enzymes. Saliva is vital for the maintenance of oral milieu and health. It is an essential diagnostic biofluid as it reflects the various systemic conditions (16). The composition of saliva, flow rate, and pH determine the important physical and biochemical properties of the oral cavity such as protection and lubrication of oral mucosal tissues, remineralization of teeth, and alimentation (17,8,26). Hence the research towards the saliometric analysis has gained importance worldwide to attain knowledge in the fields of oral biology under pathological circumstances (16,17,8,26,28).

Fabian et al. (2004) reported the presence of HSP70 in saliva might involve passive transportation via salivary glands from blood serum as similar to other blood proteins (6). Studies reported that the presence of HSP70 in saliva could be due to specific active transport mechanisms using exosomes, the membrane vesicle. Thus HSP70 release could be triggered by neuronal signals or by hormone (3,30,7). Oral pathology may also severely interfere with pregnancy outcomes. In pregnant women, periodontal disease represents a risk factor for preterm birth and low birth weight babies (22,15). Oral changes seen in pregnancy include gingivitis, gingival hyperplasia, pyogenic granuloma, and salivary changes. Sometimes, increased facial pigmentation is also seen. Pregnant women are often predisposed to gingivitis and gingival hyperplasia due to elevated circulating estrogen and its positive impact on capillary permeability (9,29,19).

Likewise, several diseases and cardiovascular conditions, in addition to various changes that occur during pregnancy have been linked with hormones. It has been suggested that the changes in the level of some hormones may play key roles in maternal susceptibility to preeclampsia (35). Progesterone is popularly called as the “hormone of pregnancy” (Bowen 2000), and it has many roles relating to the development of the fetus (36). Moreover, saliva acts as a carrier of signal molecules, which are either transported into the salivary glands directly from the blood vessels or are independently produced by the glands. During pregnancy, an increase in plasma progesterone is reflected in saliva (31). Progesterone is synthesized in the placenta, and the unbound progesterone enters the saliva via intracellular mechanisms, and the majority of progesterone in the saliva is not protein-bound (32). Therefore, the determination of progesterone in saliva may be a convenient alternative when compared to blood. Adequate production of estrogens (estradiol, estrone, estriol) and progesterone by the corpus luteum and placenta are essential for a successful pregnancy. A noninvasive method to monitor hormone levels throughout pregnancy, particularly during the first trimester, could help identify hormonal deficiencies (e.g., low progesterone) that increase the risk for miscarriage and lead to therapeutic interventions, such as progesterone supplementation, for women with unrecognized luteal insufficiency.

In another study, Karnik and his colleagues (2015) reported that the salivary flow rate and pH of saliva were lower in pregnant women than in non-pregnant women (11). Sometimes, a lower pH below 5.5 may also occur, leading to the demineralization of teeth due to hydroxyapetite crystals dissolve (14). During the third trimester of pregnancy, salivary pH and buffering capacity reach their lowest levels, which increases the risk of caries incidence with higher levels of Streptococcus mutans (27,12). Hence our study focussed on identifying biomarkers in saliva for diagnosing the pregnancy complications which may aid in the regulation of good oral hygiene which can ultimately help to prevent or reduce the severity of pregnancy complications induced oral changes and vice versa. Since, HSP70 expression was found to be correlated with the oral defensive characteristics under various periodontal complications such as gingivitis, oral ulcers, dental pulp, and periapical

inflammation, mucosal allergies, etc., the current study evaluated its expression in the saliva of normal pregnant and complicated (hypertension) pregnant women. The present study is the first of its kind used to analyze the expression of HSP70 in saliva (as a non-invasive method) of pregnant women to assess its value under pregnancy disorders to manage medically compromised women with high-risk pregnancy complications.

Saliva testing is used to screen for diagnose numerous conditions and disease states, including Cushing's disease (cortisol), metabolic disturbances (such as insulin resistance, diabetes, and metabolic syndrome) cardiovascular disease (CRP, nitric oxide), cancer (pancreatitic, breast and oral), HIV, hepatitis, parasites (Helicobacter pylori infection), hypogonadism (testosterone), and allergies. However, there is still a challenging condition persisting in the screening of pregnancy complication using biofluids of non-invasive origin like saliva. Hence, the present study aims to elucidate how pregnancy alters the oral biochemical milieu in terms of salivary HSP70 under normal and complicated pregnancy conditions, and also to reveal a non-invasive biomarker to help clinicians to better adjust their preventive strategies to minimize oral pathology and the associated pregnancy complications.

MATERIALS AND METHODS

Selection of Subjects

S.No	Particulars	
1	Period of study	**From June 2017-September 2017**
2	Sample	**Saliva**
3	Sample size (number)	**Normal: 10 and Hypertensive: 10**
4	Sample from	Normal and hypertensive patients in private hospital at Chennai **Enclosure:** Informed consent was obtained from the subjects
5	Saliva collection method	**Passive drool method** (detailed procedure given below and Figure A)
6	Storage temperature and use	Collected salivary samples were **assayed for proteins and HSP70 immediately** Remaining samples were stored at **-20ºC** immediately to preserve the sample for possible use in future studies
7	Age	**30-34 yrs**
8	Gestational period	**3rd trimester**
9	Gestational weight (body weight)	60-80 kg
10	Clinical characteristics criteria: **Blood pressure**	**Normal:** Clinically stable patients with a blood pressure lesser than or equal to 140/90mm/Hg **Preeclamptic:** More than 140/90mm/Hg
11	**Exclusion criteria** • Patients with severe preeclampsia and other severe maternal complications • Patients using saliva affecting drugs (anticholinergics, antidepressants) • Having diseases like Sjögren's syndrome and other connective tissue diseases • Previous radiotherapy or chemotherapy • Oral mucosal/gingival diseases	

12	**Precautions** **Patients are advised to** • Avoid foods with high sugar or acidity, or high caffeine content, immediately before sample collection, since they may compromise the assay by lowering saliva pH and increasing bacterial growth • Document consumption of alcohol, caffeine, nicotine, and prescription/over-the-counter medications within the prior 12 hours • Document vigorous physical activity and the presence of oral diseases or injury • Not to eat a major meal within 60 minutes of sample collection. • Rinse mouth with water to remove food residue and wait at least 10 minutes after rinsing to avoid sample dilution before collecting saliva.

Saliva Collection by Passive Drool Method (SALIMETRICS)

Initiated by removing the cap from cryovial (10x46mm, catalog no: 5002.01, Biogenuix Medsystems Pvt. Ltd, SALIMETRICS) and saliva collection aid (SCA) (catalog no: 5016.02, Biogenuix Medsystems Pvt. Ltd, SALIMETRICS), followed by inserting the SCA securely into cryovial. Then the SCA-cryovial insert was given to participants and instructed to keep the insert into the mouth and allow saliva to pool in the mouth. For the maximum sample collection, patients are advised to keep their head tilt forward and drool the saliva through the SCA to collect into the cryovial. Same was repeated until the collection of sufficient sample. Then the cryovial was removed from the SCA, closed tightly and labeled with freezer proof labels (catalog no: BMSCLABEL-2025, Biogenuix Medsystems Pvt. Ltd, SALIMETRICS).

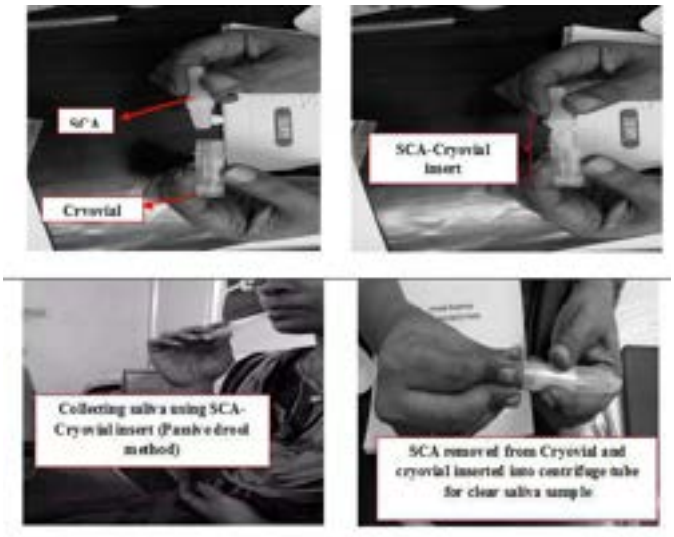


Figure A: Saliva collection and processing

Processing of Saliva Sample

Saliva samples are to be assayed, are a vortex, and then centrifuged at 1500 x g for 15 minutes. Clear saliva, avoiding any sediment present in the bottom of the tube, was utilized for the estimation of salivary proteins and HSP70. For viscous saliva, centrifugation time was increased from 15 minutes to 20 minutes.

Estimation of Protein

Protein concentration was determined by the method of Bradford (1976) (1). The Bradford assay is based on an absorbance shift in the dye Coomassie when the previously red form Coomassie reagent changes and stabilizes into Coomassie blue by the binding of the protein. About, 0.1-0.5 ml of working BSA standard of the concentration 10-50 µg was pipetted out into a series of test tubes labeled as S1-S5. Suitably diluted saliva samples (10 µl) were taken in the test tubes. The volume was made up to 1 ml with distilled water in all the tubes. 1 ml of distilled water alone served as blank. 5 ml assay reagent (Dye stock: 100 mg Coomassie brilliant blue (CBB) G-250 in 50 ml absolute ethanol and 100 ml 85% orthophosphoric acid, being diluted to 200 ml with distilled water; Assay reagent: It was prepared by diluting 1 volume of the dye stock with 4 volumes of distilled water) was added to all the tubes and incubated at room temperature for 20 min. The colour developed was diluted in the ratio of 1:4 using distilled water, and the absorbance was read at 595 nm. The protein concentration was calculated and expressed in terms of mg protein/mL of saliva samples.

Estimation of Salivary HSP70

The inducible form of HSP70 in the salivary samples was quantified using the HSP70 ELISA kit (EKS-700B,

Stressgen, Canada) according to the manufacturer's instruction. The protein was diluted using a buffer and plated along with the diluted recombinant HSP70 standard. After incubating at room temperature for 2 h, the contents were aspirated and washed with wash buffer for 4 times. Then, the wells were incubated with the HSP70 antibody for 1 h and washed with a wash buffer for 4 times. Following incubation, the wells were again incubated with HSP70 conjugate for 1 h and washed with a wash buffer for 4 times. The assay was developed with tetramethylbenzidine (TMB) substrate and incubated at room temperature for 30 min. The blue color developed was in proportion to the amount of captured HSP70. The color development was stopped with an acid stop solution, which converted the endpoint color to yellow, and the color intensity was measured in a microplate reader at 450nm. The concentration of HSP70 present in the saliva sample was calculated by plotting the HSP70 standard curve.

Estimation of Progesterone

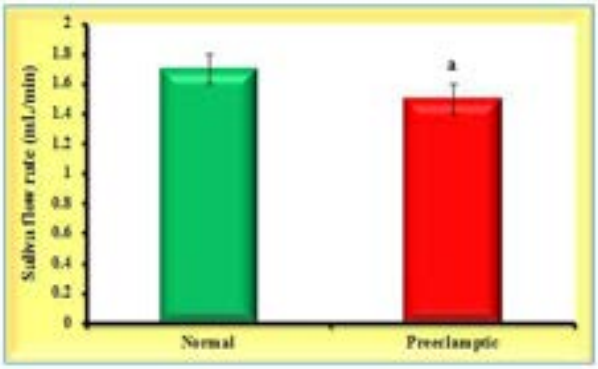
ELISA kits for salivary progesterone was purchased and used according to the kit inserts. For comparison of the performance and suitability of the kits, several extra validation experiments were performed.

Statistical Analysis

The results were expressed as mean value ± standard deviation (SD). Statistical analysis of the data was carried out using the Statistical Package for Social Sciences (SPSS) 17 version package. Statistical significance was determined by comparing the results of the preeclamptic saliva sample with the normotensive sample using Student's t-test. Differences were determined to be statistically significant for values of $p<0.05$, $p<0.01$, and $p<0.001$.

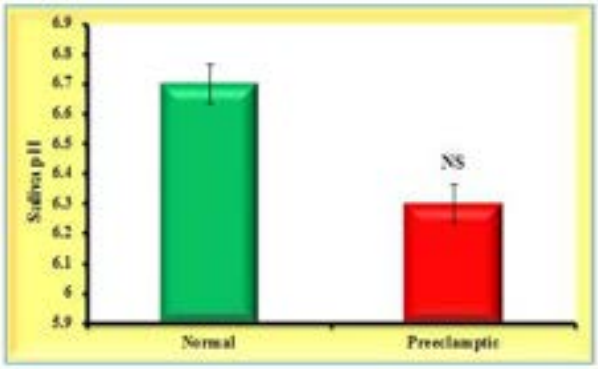
RESULTS

Figure 1: Flow rate of saliva in normal and preeclamptic pregnant women



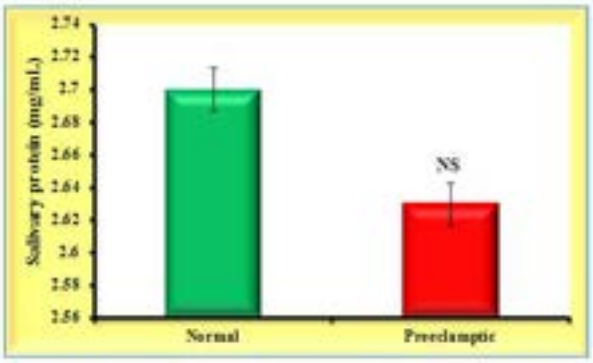
Decreased flow rate (12%, ᵃp<0.05) of saliva was observed in preeclamptic women

Figure 2: pH of saliva in normal and preeclamptic pregnant women



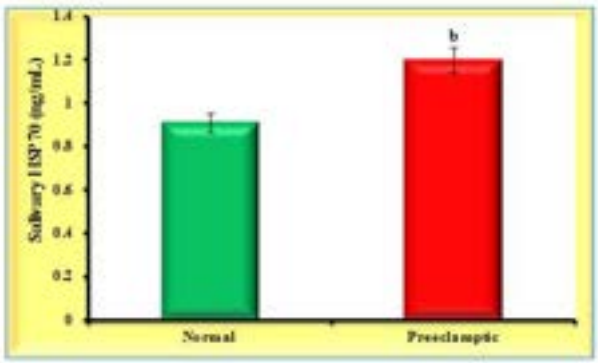
Decreased pH (5.9%, NS) of saliva was observed in preeclamptic women

Figure 3. Level of protein in saliva of normal and preeclamptic pregnant women



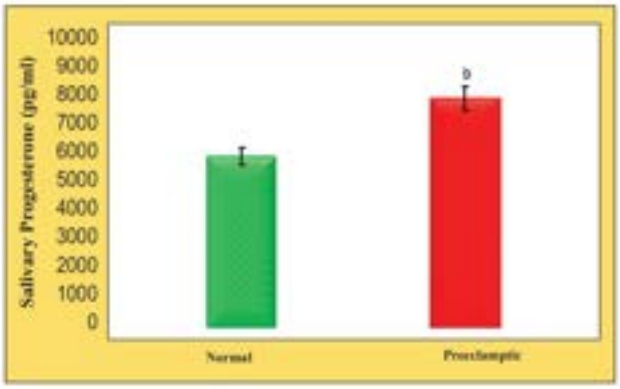
Decreased protein (3%, NS) in saliva was observed in preeclamptic women

Figure 4: Level of HSP70 in saliva of normal and preeclamptic pregnant women



Increased HSP70 (32%, $^{b}p<0.001$) in saliva was observed in preeclamptic women

Figure 5: Level of Progesterone in saliva of normal and preeclamptic pregnant women



Increased Progesterone (34%, $^{b}p<0.001$) in saliva was observed in preeclamptic women

DISCUSSION

The results of the present study reveal that the quantification of HSP70 in the saliva of pregnant women represents a suitable biomarker to diagnose pregnancy complications. Enhanced expression of HSP70 in the saliva sample of preeclamptic patients when compared to normotensive reports that HSP70 in the extracellular fluid like saliva may serve as the protective event which may elicit an innate or adaptive proinflammatory immune response to avoid the propagation of the insult. Elevated salivary HSP70 under preeclamptic condition supports the research reported by Lewthwaite et al. (2002) (13), stating that eHSP70 executes the adaptive characteristics, unlike the intracellular HSP70s which have the predominant chaperone activity under physiological stress condition.

The expression of HSP70 may be elicited either by passive transport from blood serum or by active transport using exosomes, the membrane vesicles (3,30,7,6) due to systemic stress associated oral

insult. A study by Molvarec et al. (2006) (18) reported that high serum HSP70 is a marker of hypertensive pregnancy and in other studies (23) it is reported that this overexpression in circulation is essential for the survival of critically diseased individuals. This overexpression of HSP70 in circulation is also reflected in placental cells (trophoblasts and endothelial cells) suggesting that under physiological stress condition HSP70 elevation is primary to signal the downstream signaling molecules to promote the adaptive function (20,21). Similarly, the expression of HSP70 is elevated in saliva of preeclamptic patients indicating that it represents a novel tool in determining pregnancy-induced hypertension. It also further evident that this overexpression may be responsible for the preterm but live fetal delivery along with the conservation of maternal life. Hence, the assessment of HSP70 in the saliva of pregnant women may aid in the preliminary diagnosis of pregnancy complications to avoid aberrant complexity.

Earlier studies have reported that in a pregnant woman suffering from the HELLP syndrome, the levels of salivary progesterone were very high. The relation between progesterone and high blood pressure has been described in the literature and measuring progesterone in saliva during pregnancy may give indications of this syndrome early in the third trimester and time to take measures. Evidence shows that the function of the steroid hormone receptors is regulated by a group of constitutively synthesized heat shock proteins (HSPs) (37). HSPs, or stress proteins, are endogenous proteins that are either present constitutively, functioning as chaperones (38), or induced upon cell stress, such as heat, oxidative stress, ischaemia, and hypoxia (39). The proposed mechanism that links an elevation of HSP70 in myometrium and spontaneous labor (33) is that the intra-cellular HSP70, by binding to the progesterone receptor, functions as a co-repressor of this receptor and suppresses progesterone binding to the nuclear response element (34).

Since it is a non-invasive technique involved in the sample collection of large quantities with relatively stress-free protocol, further substantial future research is required to standardize saliva collection techniques to validate salivary biomarkers and establish reference ranges, before it is practiced as a diagnostic technique for pregnancy complications.

CONCLUSION

Significantly increased expression of HSP70 and Progesterone in saliva samples of preeclamptic patients conclude that the expression of HSP70 and Progesterone in saliva can be used as an excellent non-invasive diagnostic marker for screening pregnancy-induced hypertensive disorders.

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Appendix 1

Maternal Anthropometry

H1 Weight ---------- Kg

H2 Height ---------- cm

H3 MUAC (Mid upper arm circumference) ---------- cm

Health questionnaire: pregnant woman

Name:

Today's date:

Weight:

Height:

Weight before this pregnancy:

Due date

1. Please describe your health and your pregnancy.

2. a. Is this your first pregnancy?

□ Yes (skip to question 3)

□ No (please answer questions b. through e. below)

b. For births after 20 weeks, were any stillbirths or neonatal deaths?

□ Yes □ No

c. Were any born at or before 37 weeks?

□ Yes □ No

d. Were any born less than or equal to 2.5 kg ?

□ Yes □ No

e. What was the date your last pregnancy ended?

3. Do you have prenatal care for this pregnancy?

□ Yes, I started prenatal care in the month of pregnancy.

□ No

4. Have you had any medical problems with this or any pregnancy?

□ Yes (please list)

□ No

5. Do you take any medications now?

□ Yes (please list)

□ No

6. Do you smoke cigarettes now?

□ Yes. How many per day?

□ No

7. Does anyone living in your household smoke inside the home?

□ Yes □ No

8. During this pregnancy have you had any beer, wine or hard liquor?

□ Yes. How many drinks do you have per week?

□ No

9. Have you used any drugs during this pregnancy?

□ Yes □ No

10. Are you willing to provide saliva sample for biochemical analysis?

□ Yes □ No