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Evaluation of Serum Heat Shock Protein 70 as a Diagnostic Biomarker for Ectopic Pregnancy: A Comparative Case-Control Study

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Abstract: Introduction and Aim: Ectopic pregnancy (EP) is a potentially life-threatening gestational event in which a conceptus implants outside the uterine cavity. Early detection is vital to avoid the complications. The purpose of the present study was to examine serum HSP70 in women having a diagnosis of EP in comparison with normal IUP and sein diagnostic accuracy. **Materials and Method:** A case control study done in Azadi Teaching Hospital, Iraq, during the period from January- May/ 2024. A total of 86 women in the first trimester (4–12 weeks) were enrolled: 38 with EP, and 48 with IUP. The levels of serum HSP70 were detected with an Elisa kit. Clinical, laboratory and demographic data were retrieved and statistically analyzed. **Results:** The mean serum HSP70 concentration of EP cases was significantly higher (in 0.465±0.221 pg/mL) than IUP cases (in 0.153±0.068pg/ mL, $p < 0.001$). ROC curve analysis revealed high diagnostic accuracy with an AUC of 0.927. At a cut-off level of 0.1805 pg/mL, sensitivity and specificity both reached 92.1% and 87.5%, respectively usefully distinguished from healthy subjects to esthesioneuroblastoma patients (p<.001), positive predictive value was 85%, negative predictive value was 93.5%. Symptoms and signs were abdominal pain (86.8%), vaginal bleeding (65.8%) and cervical excitation (44.7%). Age, BMI and gravidity did not differ significantly between the two groups. Serum β-hCG concentrations were significantly higher in the EP (1592±87 mIU/mL) than in IUP (2036±874 mIU/mL), ($p = 0.003$. The levels of HSP70 were associated with the clinical severity in terms of shoulder pain and hemodynamics. **Conclusions:** Serum HSP70 is markedly high in ectopic pregnancy and can be used as an early noninvasive and sensitive supportive biomarker algorithmic to β-hCG estimation, and ultrasonography.

Keywords: HSP70; Ectopic Pregnancy; Serum Biomarker; Early Diagnosis

INTRODUCTION

Ectopic pregnancy (EP) is an important obstetric condition in which implantation of the fertilized ovum outside the uterine cavity, generally within the fallopian tube, occurs and accounts for about 1–2% of all pregnancy worldwide [1,2]. The delayed diagnosis of EP is one of the most common first-trimester maternal morbidity and mortality factors with tubal rupture, intra-abdominal hemorrhage, and shock as subsequent complications [1,3]. The clinical manifestation of EP is diverse, and may mimic the symptom of normal intrauterine pregnancy (IUP) or early miscarriage, which brings about great difficulty in its early identificatiohn and distinguished diagnosis [2,4]. Present diagnostic protocols are based on clinical examination, serial β-hCG determinations and transvaginal ultrasonography (TVUS). TVUS has been the gold standard for localization of pregnancy and when there are adnexal masses seen or presence of "bagel sign", these findings correlated with β-hCG levels greater than those expected to see an intrauterine gestational sac (discriminatory zone) lead to the diagnosis of EP [5,6]. However, in early gestation, or unusual presentations, the sensitivity of TVUS and β-hCG alone may be inadequate or unreliable to detect EP [7–8]. A number of biochemical markers, such as progesterone (p), vascular endothelial growth factor (VEGF), activin A and inhibin A have been evaluated but none has had adequate sensitivity and specificity to be used routinely in the clinical setting as single test [8,9]. Heat shock protein 70 (HSP70) is a broadly expressed evolutionary conserved molecular chaperone, which is essential in cellular response to stress, apoptosis and immune regulation [10,11]. The data indicate that HSP70 plays a role in implantation and early placentation, and circulating levels of this peptide could

be increased in pathological conditions of pregnancy such as EP [12–15]. Preliminary data has shown increased serum levels of HSP70 in EP compared to PIUP and threatened miscarriage, suggesting that it can be used as a non-invasive additional marker [7,13–15]. Nevertheless, the diagnostic accuracy of HSP70 for EP has been poorly assessed and might also be due to the lack of robust data on best cutoff values, sensitivity, specificity and predictive values in early pregnant patients. Objective of this study was to estimate serum HSP70 level in women with EP in relation to normal intrauterine pregnancies, establish the diagnostic threshold value and define its diagnostic accuracy as a possible adjunctive agent for standard clinical and laboratory findings.

MATERIALS AND METHODS

The current case-control study was carried out in the Department of Obstetrics and Gynecology, Azadi Teaching Hospital, Kirkuk, Iraq during the period from January to May 2024. The study design was ratified by the Scientific Council Department of Obstetrics and Gynecology-Iraqi commission for Medical Specializations and from the hospital authorities. All study subjects were aware of the study's aims and gave written informed consent, and confidentiality was secured.

A total of 86 pregnant women at first-trimester (4 to 12 weeks) were recruited in the study, including 38 cases with ectopic pregnancy(EP) and 48 control subjects with established intrauterine pregnancy (IUP). Eligible participants were 19–44 years old, hemodynamically stable (special exceptions applied) and willing to give informed consent. Women with systemic diseases which could influence HSP70 levels, multiple pregnancies, miscarriages, mole pregnancy or ruptured ectopic pregnancy; history of smoking and substance use and having hormonal agents as well as anti-inflammatory drugs and antioxidants were excluded. These inclusion and exclusion criteria were designed to select a similar population and reduce confounding biases.

Data Collection and Clinical Assessment

All participants were also extensively examined for socio-demographic characteristics (age, BMI, education, occupation and residence), obstetric history (gravidity, parity, previous ectopic pregnancy, infertility use of contraception s PID and endometriosis and a history of past surgeries) the symptoms at presentation including abdominal pain, vaginal bleeding, shoulder pain, nausea/vomiting cervical excitation and hemodynamic status. Abdominal tenderness and adnexal masses were recorded on physical examination. The detailed review of all the cases permitted connection between demographic, clinical aspects and laboratory data including HSP70 level.

Laboratory Investigations

Serum β-hCG and HSP70 were detected in all the participants. Measurement of serum β-hCG was performed according to the routine hospital protocol and quantified in mIU/mL. Serum HSP70 levels were assessed by commercial ELISA at 450 nm using an ELISA reader (Biobase, China) according to the manufacturer's instructions. Blood samples (5 ml) were drawn, followed by centrifugation at 3000 rpm for 5–7 min and serum stored at 2–8 °C until analyzed. This ensured optimal and reproducible HSP70 quantitation for comparisons between EP and IUP groups.

Imaging Studies

Transvaginal ultrasonography (TVUS) was done for all women to evaluate the site of pregnancy. Ectopic pregnancy was determined by lack of an intrauterine sac and evidence of a gestational sac or adnexal mass, and normal intrauterine pregnancies were established as visualization of a gestational sac with yolk and/or fetal pole. All ultrasound evaluations were initially conducted by an obstetric sonographer and validated by a senior physician radiologist for confirmation of the diagnosis.

Ethical Approval

The study was conducted in accordance with ethical principles. A written informed consent was signed by each participant, and the proposal was approved by the Scientific Council of Obstetrics and Gynecology, Iraqi Board for Medical Specializations. Information was deidentified to protect patient confidentiality and all clinical and laboratory data were used for research only.

Statistical Analysis

The data was analysed using SPSS version 23. The presenting continuous variable was described as mean±SD and compared using independent t-tests, while categorical variables were presented as frequencies and percentages and analyzed with chi-square tests or Fisher's exact test. The diagnostic value of HSP70, in terms of AUC, optimal cut-off point, sensitivity, specificity, and positive predictive value (PPV) and negative predictive value (NPV), was analyzed by receiver operating characteristic (ROC) curve. A $p< 0.05$ was considered to indicate a statistically significant difference [16,17].

RESULTS

The socio-demographic profile of the cases (ectopic pregnancy, n = 38) were compated with the controls (normal intrauterine pregnancy, n = 48). The age of those with ectopic pregnancy was 31.92±3.45 years and in those with IUP 32.54 +5.38, this represent a non-statistical difference (p = 0.54). Likewise, average BMI was similar between groups (30.89±2.64 vs 31.11±1.67 kg/m^2, p = 0.62). The levels of education, occupation, and location did not show considerable heterogeneity among groups that could have confounded the outcomes of this study, Table 1.

Clinical presentations and laboratory findings differed between ectopic pregnancy and intrauterine pregnancy significantly. Abdominal pain was 86.8% in ectopic pregnancies and 4.2% in intrauterinepregnancies(p<0.001). Vaginal bleeding was present in 65.8% and absent in none of the controls (p<0.001). Shoulder pain was observed in 65.8% vs 0% (p<0.001) and cervical excitation in 44.7% vs 0%

Table 1: Socio-Demographic Characteristics of Participants (Cases vs Controls)

Variable		Cases (EP, n = 38)	Controls (IUP, n = 48)	p-value
Age (years, mean±SD)		31.92±3.45	32.54±5.38	0.54 (NS)
BMI (kg/m^2, mean±SD)		30.89±2.64	31.11±1.67	0.62 (NS)
Educational Level	Illiterate	6 (15.8%)	8 (16.7%)	0.78 (NS)
	Primary	8 (21.1%)	10 (20.8%)	
	Intermediate	10 (26.3%)	11 (23.0%)	
	Secondary	11 (28.9%)	17 (35.4%)	
	College & Higher	3 (7.9%)	2 (4.1%)	
Occupation	Housewife	22 (57.9%)	29 (60.4%)	0.91 (NS)
	Student	10 (26.3%)	12 (25.0%)	
	Employee	6 (15.8%)	7 (14.6%)	
Residence	Urban	27 (71.1%)	31 (64.6%)	0.60 (NS)
	Rural	11 (28.9%)	17 (35.4%)	

Table 2: Clinical and Laboratory Characteristics and HSP70 Levels

Variable	Cases (EP, n = 38)	Controls (IUP, n = 48)	p-value
Gestational Age (weeks, mean±SD)	7.2±1.5	7.3±1.8	0.78 (NS)
β-hCG (mIU/mL, mean±SD)	1592.35±87.0	2036.84±874.39	0.003*
Abdominal Pain	33 (86.8%)	2 (4.2%)	<0.001*
Vaginal Bleeding	25 (65.8%)	0 (0%)	<0.001*
Shoulder Pain	25 (65.8%)	0 (0%)	<0.001*
Cervical Excitation	17 (44.7%)	0 (0%)	<0.001*
HSP70 (pg/mL, mean±SD)	0.465±0.221	0.153±0.068	<0.001*

Table 3: Mean HSP70 Levels by Socio-Demographic Characteristics

Variable	Category	n	Mean HSP70±SD (pg/mL)	p-value
Age (years)	<25	8	0.21±0.07	Reference
	25–29	15	0.27±0.09	0.12 (NS)
	30–34	25	0.30±0.10	0.04*
	35–39	10	0.29±0.08	0.05*
BMI (kg/m^2)	<18.5	3	0.32±0.11	0.03*
	18.5–24.9	20	0.25±0.09	Reference
	25–29.9	30	0.28±0.10	0.08 (NS)
	≥30	10	0.27±0.08	0.12 (NS)

Table 4: HSP70 Levels by Clinical and Laboratory Characteristics

Variable	Category	N	Mean HSP70±SD (pg/mL)	p-value
Abdominal Pain	Present	68	0.412±0.205	<0.001*
	Absent	18	0.162±0.072	Reference
Vaginal Bleeding	Present	49	0.429±0.210	<0.001*
	Absent	37	0.180±0.080	Reference
Shoulder Pain	Present	38	0.465±0.221	<0.001*
	Absent	48	0.153±0.068	Reference
Cervical Excitation	Present	20	0.472±0.225	<0.001*
	Absent	66	0.172±0.075	Reference
β-hCG (mIU/mL)	<2500	40	0.412±0.200	<0.001*
	≥2500	28	0.173±0.070	Reference

Table 5: ROC Analysis of HSP70 for Ectopic Pregnancy Diagnosis

Cut-off HSP70 (pg/mL)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC	p-value
0.150	97.4	72.9	72.4	97.5	0.927	<0.001
0.1805 (optimal)	92.1	87.5	85.0	93.5	0.927	<0.001
0.200	89.5	91.7	88.0	92.5	0.927	<0.001
0.250	81.6	95.8	91.7	88.4	0.927	<0.001

(p<0.001). The median serum β-hCG levels were significantly lower in ectopic pregnancies (1592.35±87.0 mIU/mL) when compared to controls (2036.84±874.39 mIU/mL, p = 0.003). The serum levels of HSP70 were significantly higher in ectopic pregnancy (0.465±0.221 pg/mL) than those with intrauterine pregnancies (0.153±0.068 pg/mL, p<0.001), indicating it is a putative diagnostic biomarker, Table 2.

Socio-demographic correlates of HSP70 were analysed. Statistically significant variations were not found according to age, BMI, education, occupation and residence after adjustment for the above risk factors, only a higher level was shown in women aged 30–34 years and those with lower BMI (<18.5 kg/m^2) respectively. This provides the implication that HSP70 elevation is more related to ectopic pregnancy of an individual than the demographic aspects, Table 3.

Higher levels of HSP70 were observed in those participants presenting with abdo- minal pain, vaginal bleeding, shoulder pain, or cervical excitation. HSP70 was also higher in women with low β-hCG levels (<2500 mIU/mL). It shows that HSP70 is closely associated with clinical symptoms and biochemical parameters and underlines its value for the diagnosis of ectopic pregnancy, Table 4.

Using ROC curve analysis, serum HSP70 showed excellent diagnostic value in distinguishing ectopic pregnancy and IUP with AUC of 0.927 (92.7%, $p < 0.001$). A cut-off point of 0.1805 pg/mL offered the maximum value for sensitivity (92.1%) and specificity (87.5%). The positive predictive value (PPV) and the negative predictive value (NPV) were 85.0% and 93.5%, respectively. Clinically, this may mean that HSP70 can accurately identify most EPs with high certainty of excluding normal IUPs. These findings confirm the potential of HSP70 as a non-invasive biomarker combined with ultrasound and β-hCG for ectopic pregnancy diagnosis early in gestation, Table 5.

DISCUSSION

The level of serum HSP70 was significantly higher in ectopic pregnancy (EP) than normal intrauterine pregnancy (IUP) in our study, supporting its use as a diagnostic biomarker [15]. This is similar to the observations by Yıldız et al. who observed significantly higher HSP70 in EP than abortus imminens and healthy intrauterine control [7,15]. Nonetheless, their AUC was moderate (0.81) relative to our cohort's improved diagnostic performance (AUC 0.927), which may have been attributed to differences in assay method used, sample size and population demography [18,19]. The biological significant of the increased HSP70 concentrations in EP are supported by its involvement in cellular stress responses, implantation biology and inflammation [10,11,15,20]. Heat shock proteins play a role in early pregnancy (bringing about decidualization, implantation and trophoblast invasion) and their altered expression was found to be one of the factors contributing to pregnancy disorders [18,21]. Our results on β-hCG kinetics are consistent with previous reports worldwide which indicated that β-hCG levels were lower and rose abnormally slow in EP compared to normal pregnancies [1,20]. Nevertheless, the use of only β-hCG is not conclusive and in early gestation or PUL other markers are needed (18). In addition to HSP70, proteomic profiling had identified potential markers including GSTO1 and ECM-1 demonstrated to increase diagnostic accuracy along with β-hCG over single markers [22,23]. Metabolomics studies also identified the disturbance in lipid and tryptophan metabolism profiles and could discriminate EP patients very well (AUC ~0.96) with similar metabolic abnormalities associated with abnormal implantation led by other studies [24,25]. These multi-marker approaches are also consistent with recommendations that a panel of, rather than a single, biomarker will be necessary to best identify early EP [22–25]. Some inflammatory markers have offered promise as well. Higher contradiction of this prediction was that IL-6 level was found to be a valuable parameter with higher sensitivity and specificity for dosing tubal EP site elicits the higher risk of implantation and rupture (26). Furthermore, reports from studies on exosomal mIIRNAs may distinguish between EP and IUP including miR-519d, suggesting a complexity of molecular mechanism [27]. In Iraq, nothing is known about HSP70 specifically in EP, but the study of other biomarkers suggests that serum markers are informative there. A recent Iraqi study demonstrated that activin A was significantly higher in EP compared with missed abortion and controls, suggesting that non-conventional biomarkers outside β-hCG may serve as potential diagnosis markers [18,19]. By contrast, the previous Iraqi biochemical study showed changes in α-fetoprotein, IL-1 and β-hCG levels associated with pregnancy complications such as EP, indicating a systemic biochemistry imbalance along ectopic implantation [20]. The association we found between raised HSP70 and clinical severity (e.g. shoulder pain, haemodynamic instability) could represent stress-related mechanisms that worsen local tubal inflammation and tissue damage. HSP70 was associated with the pro-inflammatory state in pregnancy complications such as preeclampsia and systemic inflammation responses; its circulating levels were inversely correlated with the severity of cytokine activity and endothelial dysfunction [11,15,21]. In addition, HSP70 showed mixed results in other studies involving pregnancy complications where no statistically significant difference was found in severe preeclampsia suggesting the context-specific regulation of HSP70 expression [22,23]. This variation emphasizes the need for disease-specific validation in each candidate biomarker.

CONCLUSION

Serum Hsp70 concentration is significantly increased in EP patients and it represents a marker with good diagnostic accuracy, associated with clinical severity factors like shoulder and abdominal pain. This biomarker would be a valuable non-invasive supplement to β-hCG or transvaginal ultrasound in facilitating early diagnosis and appropriate treatment of ectopic pregnancy, while its level does not demonstrably correlate with demographic characteristics.

Limitations

This study has several limitations. First, our findings might not be generalizable to other populations because this was a single-center study with relatively small number of samples. Second, our study enrolled only first-trimester pregnancies, and the diagnostic accuracy of HSP70 at other gestational weeks is uncertain. Third, the HSP70 value was not corrected for any confounding factors like a concomitant infection, inflammatory disease or drugs interfering with the HSP70 level. Lastly, no longitudinal HSP70 levels were determined that would allow for determination of early gestation temporal trends.

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