O r i g i n a l i n v e s t i g a t i o n s / c o m m e n t a r i e s

Kisspeptin as a biomarker of spontaneous abortion in the first trimester of pregnancy

Aya Mohamed Selmy[1], Laila Ahmed Rashed[2], Doha El-Sayed Hamed Ellakwa[3]

[1]Department of Biochemistry and Molecular Biology, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt. [2]Department of Medical Biochemistry and molecular Biology, Faculty of Medicine, Cairo University, Cairo, Egypt. [3]Department of Biochemistry and Molecular Biology, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt.
*Corresponding Author: Aya Mohammed Selmy, Email: Ayona3368@gmail.com.

Abstract. A highly accurate serum marker for predicting viable pregnancy needs to be developed. Kisspeptin is a recently identified hormone encoded by the KISS1 gene, playing a critical role in human reproduction. Plasma kisspeptin levels rise dramatically during normal pregnancy due to placental synthesis, which implicates it as a potential tool for assessing risks of pregnancy complications. It is also a hypothalamic neuropeptide, expressed peripherally in pancreatic cells, liver and adipose tissue suggesting its role in regulating metabolic homeostasis. Spontaneous abortion is the most common complication of early pregnancy, affecting up to 20% of recognized pregnancies. This study was designed to evaluate and compare the serurm levels of kiss1 in first trimester pregnant women with normal pregnancy as well as with miscarriage to detect the potential role of kisspeptin as a putative biomarker of pregnancy loss. This was a prospective cohort study conducted on a total of 90 pregnant woman in the first trimester of pregnancy equally divided into three groups, group A, 30 women with recent spontaneous abortion, group B, 30 women complicated with threatened abortion (placental bleeding) and group 30 women with normal pregnancy as a control group. The maternal plasma in all groups was analyzed for the expression levels of KISS1 using plymerases chain reaction (PCR). We found a statistically significant correlation between the relative expression of kisspeptin and pregnancy viability. Conclusion: kisspeptin might be an independent biomarker of spontaneous abortion.

Introduction

Miscarriage or spontaneous abortion (SAB) affects 10%–20% of clinically recognized pregnancies and is most common before 12 weeks of gestation [1]. In modern practice, transvaginal ultrasonography and serial quantitative serum assessment of beta-human chorionic gonadotropin (HCG) have long been used as the accepted tools for measuring fetal viability [2]. However, approximately 20% of the cases resulting in miscarriage are also associated with increasing levels of serum beta-HCG, which are typical of a viable pregnancy, and their clinical utility is limited [3]. So, there is both a delay and high degree of uncertainty in diagnosing miscarriage using those techniques [4]. Kisspeptins are the peptide products of the KISS-1 gene, which was first identified in 1996 in Hershey, Pennsylvania (USA) as a human metastasis suppressor gene[5].they exert biological action by binding with the G-protein coupled receptor 54 (GPR54), also known as the KISS-1 receptor (KISS-1R) [6]. Kisspeptins and KISS-1R participate in different biological processes due to their expression in various tissues, including the pancreas, adipose tissue, liver, small intestine, brain, hypothalamus, adrenal glands, testes, as well as smooth muscles cells of the aorta, coronary artery and umbilical vein [7]. In placental tissues, KISS-1 mRNA and kisspeptins have been previously detected in syncytiotrophoblast and, to a lesser degree, in cytotrophoblast, whereas KISS-1R has been found to be expressed in syncytiotrophoblast, villous and invasive extravillous trophoblast [8]. The KISS1/KISS1 gene encodes 145-amino-acids prepropeptides, with the first 19 amino acids contributing to signaling peptides. Preprohormones are sent to the endoplasmic reticulum and then cleaved into four biologically active peptides that are distinguished by the number of their amino acids kisspeptin-10, kisspeptin-13, kisspeptin-14, and kisspeptin-54 (52 in rodent animals). All these peptides have a C-terminal region containing an Arg–Phe–NH2 radical, which allows these peptides to bind to and fully activate Kisspeptin receptors (KISS1R)[9]. In women, kisspeptin regulates several mechanisms, including follicular development, oocyte formation, ovulation, ovarian steroidogenesis, and embryonic and placental implantation [10]. In this last mechanism, kisspeptin plays a role in controlling cell invasion, altering its motility and adhesion [11]. The process of implantation depends on the degree of trophoblast infiltration into the uterine extracellular matrix. In this process, kisspeptin, along with other pro inflammatory cytokines, controls the trophoblastic invasion rate by stimulating apoptosis, ensuring that placentation occurs in a controlled and sequential manner [12]. In placental tissues, KISS-1 mRNA

and kisspeptins have been previously detected in syncytiotrophoblast and, to a lesser degree, in cytotrophoblast, whereas KISS-1R has been found to be expressed in syncytiotrophoblast, villous and invasive extravillous trophoblast [13]. In Vitro experimental studies have shown decreased expression of kisspeptin/GPR54 in women with recurrent spontaneous abortion, thus suggesting that kisspeptin is engaged in the embryo implantation process [14]. During pregnancy, a higher expression of kiss1 receptors (KISS1R) is observed in the first trimester and a decrease in the third trimester [15].

Subjects and Methods

1) Study population

This study is an experimental prospective case-control pilot study, including ninety women subjects. The study was approved by the Ethics Committee of the Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt. The study was conducted in the period from January 2018 to April 2021. Informed consent was obtained from the study participants. The samples were recruited from the Obstetrics and Gynecology Department, Faculty of Medicine, Al-Azhar University, Cairo. A total of 90 women in the first trimester of pregnancy were enrolled in three subject groups, group A (n=30, women with recently confirmed spontaneous abortion), group B (n=30, women suffered from placental bleeding (threatened abortion) and group C/ control group (n=30, uncomplicated pregnancy women). The gestational age of the study women ranged from 7 to 12 weeks of pregnancy, and it was measured clinically depending on the first day of the last menstrual cycle. Enrolled subjects had an age range from 23 to 44 years, with singleton, intrauterine pregnancy. Exclusion criteria were as follows: (a) history of medical illness (diabetes mellitus, hypertension, renal failure, or hepatic, cardiac, or autoimmune diseases), (b) ectopic pregnancy, (c) gestational age >12 weeks at the time of blood sampling , (d) abortion due to infection or trauma . All patients were subjected to the following: full history taking including, personal history, present history, obstetric history, and menstrual history. All patients were subjected to general examination; including vital signs: pulse, blood pressure and temperature and body mass index (weight in Kg/m2). General examination also included upper and lower limps, chest, heart and abdominal examination. Local pelvic examination including: Inspection of the perineum for amount of bleeding, signs of trauma or lesions to detect any abnormalities, infections or ulcer. Speculum examination to localize the bleeding origin: vagina, cervix, uterus; quantify the bleeding; inspect the cervix for presence of polyps, ulcers or other lesions. Routine antenatal investigations (complete blood count, cytomegalovirus antibodies, Rh ABO, rubella antibodies, hepatitis B surface antigen, fasting and postprandial blood sugar and complete urine analysis).

2) Plasma collection

5ml blood of venous blood of each participant was withdrawn under sterile conditions into commercially available EDTA tubes. The blood samples were centrifuged at 1800xg for 10 and the supernatant was separated and filtered through a 0.2 mm pore size membrane. Collected plasma samples were then stored at -80°C for later use.

3) Gene expression analysis

3.1. RNA isolation and cDNA synthesis Total RNA was extracted from blood plasma using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA concentration and purity were measured using a NanoDrop ND2000 spectrophotometer (Thermo Scientific, USA). Extracted RNA was reverse transcribed into complementary DNA (cDNA) using SensiFASTTM cDNA Synthesis Kit (Bioline, Germany) according to the manufacturer's Instructions. Reactions were done under the following cycling conditions: 25 °C for 10 min (annealing step), 42 °C for 15 min (reverse transcription step), 85 °C for 5 min (inactivation step) then holding at 4 °C, using Perkin Elmer thermal cycler (Applied Biosystems).

3.2. Quantitative real-time polymerase chain reaction (qRT-PCR)

Gene expression analysis was performed using Power Up SYBR green PCR Master Mix (Applied Biosystems) using the following primers

Kisspeptin 5`-GTAGATCCAACTCACTGGTTTCGTGGCAG- 3` and 5`- GCTAAGCTTTCACTGCCCCGCACCTG - 3`
Cyclophilin A 5`-CCCACCGTGTTCTTCGACAT3` and 5`- CCAGTGCTCAGAGCACGAAA-3. Reactions were performed in an ABI 7500 detection system (Applied Biosystems) with the following cycling conditions: initial activation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 15s followed by annealing and extension step at 60°C for 1 min. PPIA was expressed in all conditions confirming the integrality of RNA. After normalization to PPIA expression levels, gene expression was calculated by the comparative ct method for relative quantification $2^{-\triangle\triangle CT}$ (Livak and Schmittgen, 2001)[16]

4) ultrasound assessment

Ultrasound examinations were performed by experienced sonographers only, using a Voluson E8 or E10 system (GE Medical Systems, Zipf, Austria). 3D ultrasound examinations were obtained using a transvaginal 6–12

MHz transducer. Vasculrity of the complete placenta and embryo was imaged using power Doppler ultrasound (PD US). Every case was asked to hold their breath for approximately 30 seconds during image aquisition to minimize artifacts and measurement errors by movement. All ultrasound examinations were performed according to international guidelines on safe use of Doppler ultrasound in the first trimester of pregnancy.

Statistical analysis:

Recorded data were analyzed using the statistical package for social sciences, version 23.0 (SPSS Inc., Chicago, Illinois, USA). The quantitative data were presented as mean± standard deviation and ranges. Also qualitative variables were presented as number and percentages.

The following tests were done:

- *A one-way analysis of variance (ANOVA)* when comparing between more than two means.

- *Post Hoc test: Tukey's test* was used for multiple comparisons between different variables.

- The Comparison between groups with qualitative data was done by using *Chi-square test* and *Fisher's exact test* instead of Chi-square test only when the expected count in any cell less than 5.

- *Spearman's rank correlation coefficient (rs)* was used to assess the degree of association between two sets of variables if one or both of them was skewed.

- *Positive* = Increase in the independent variable leads to increase in the dependent variable.

- *Negative* = Increase in the independent variable leads to decrease in the dependent.

- *Scatter plot:* a graph in which the values of two variables are plotted along two axes, the pattern of the resulting points revealing correlation present.

- *The confidence interval was set to 95%* and the margin of error accepted was set to 5%. So, the p-value was considered significant as the following:

- Probability (P-value)
  - P-value ≤ 0.05 was considered significant.
  - P-value ≤ 0.001 was considered as highly significant.
  - P-value > 0.05 was considered insignificant.

Results

The results of the present study are demonstrated in the following tables and figures.

Table 1. Comparison between all the studied groups according to demographic data

Demographic data	Group A (Abortion) *(n=30)*	Group B (threatened /Bleeding) *(n=30)*	Group C (Control) *(n=30)*	F-test	p-value
Maternal age Mean±SD Range	34.60±6.69 24-44	34.40±6.54 23-43	31.50±4.74 26-40	0.961	0.414
Gestational age (weeks) Mean±SD Range	9.20±1.69 7-12	8.80±1.03 8-10	8.40±1.07 7-10	1.108	0.368
Gestational diabetes Negative Positive	18 (60.0%) 12 (40.0%)	12 (40.0%) 18 (60.0%)	24 (80.0%) 6 (20.0%)	3.900	0.174

Table 2 . Comparison between all the studied groups according to Body mass index (BMI) "Kg/m2".

Body mass index (BMI) "Kg/m2"	Abortion cases (n=30)	Bleeding cases (n=30)	Control cases (n=30)	Test value	P-value	Multiple Comparison		
						P1	P2	P3
Mean±SD	28.90±3.54	28.00±3.40	21.10±2.25	56.22	<0.001**	0.267	<0.001**	<0.001**

| Range | 22-35 | 23-34 | 18-25 | | | | | |

Table 3 .Comparison between all the studied groups according to Blood pressure (BP) "mg/dl".

Blood pressure(BP) "mg/dl"	Abortion cases (n=30)	Bleeding cases (n=30)	Control cases (n=30)	Test value	P-value	Multiple Comparison		
						P1	P2	P3
Mean±SD	143.00±15.79	126.00±11.77	90.00±14.38	110.8	<0.001**	<0.001**	<0.001**	<0.001**
Range	120-170	105-145	70-110					

Table 4. Comparison between all the studied groups according to Kisspeptin relative expression

Kisspeptin	Abortion cases (n=30)	Bleeding cases (n=30)	Control cases (n=30)	Test value	P-value	Multiple Comparison		
						P1	P2	P3
Mean±SD	0.20±0.07	0.53±0.17	1.02±0.01	447.4	<0.001 **	<0.001 **	<0.001 **	<0.001 **
Range	0.11-0.33	0.27-0.79	1-1.04					

Figure 1. Comparison between all the studied groups as regard
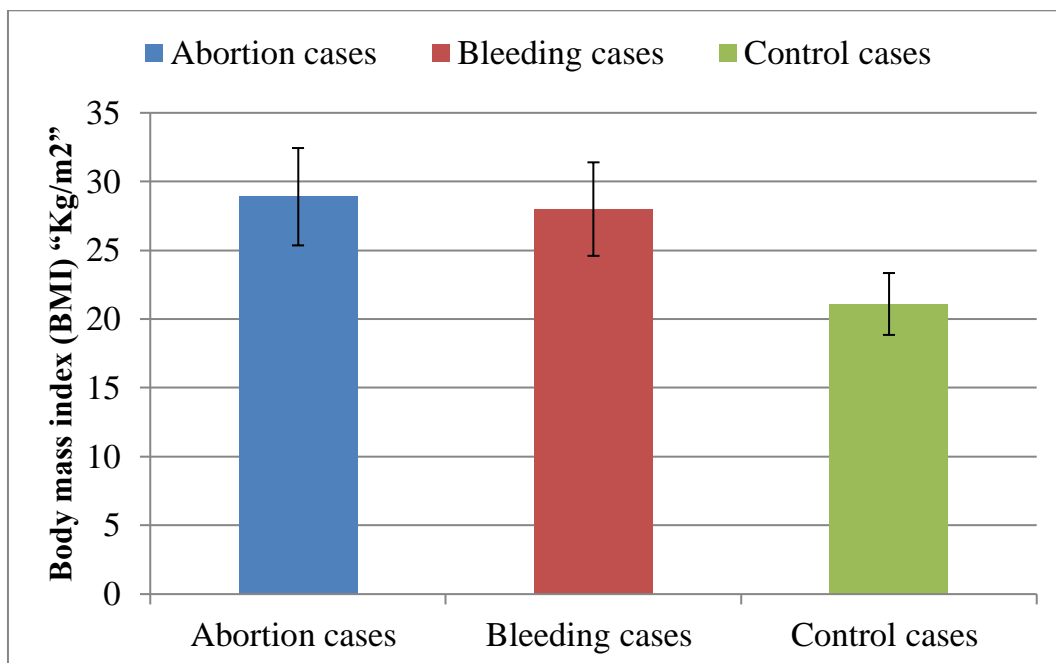Body mass index (BMI) "Kg/m2".

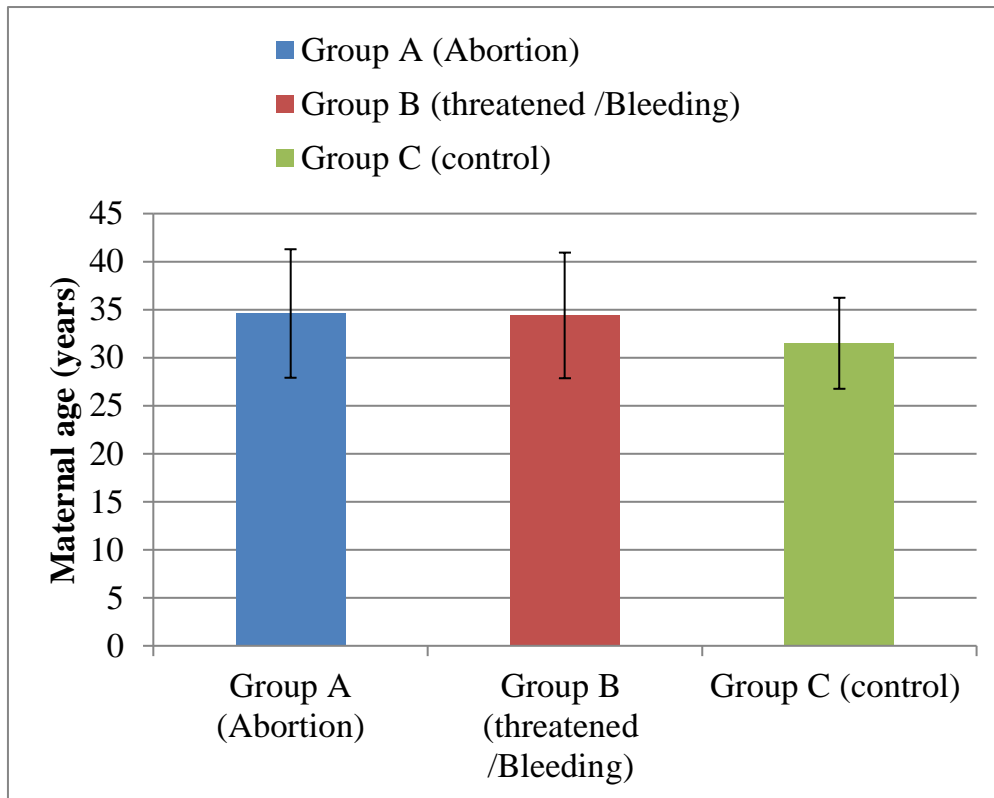**Figure 2.** Comparison between all the studied groups as regard Maternal age.

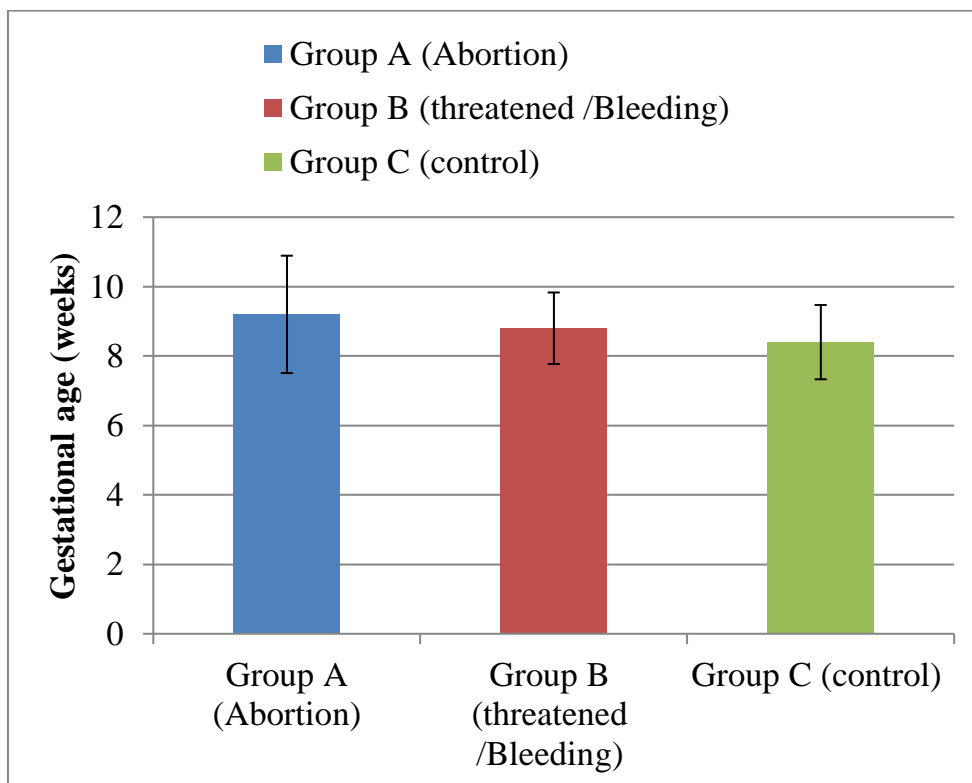**Figure 3.** Comparison between all the studied groups according to Gestational age "wks.".

Figure 4. Comparison between all the studied groups according to gestational diabetes.
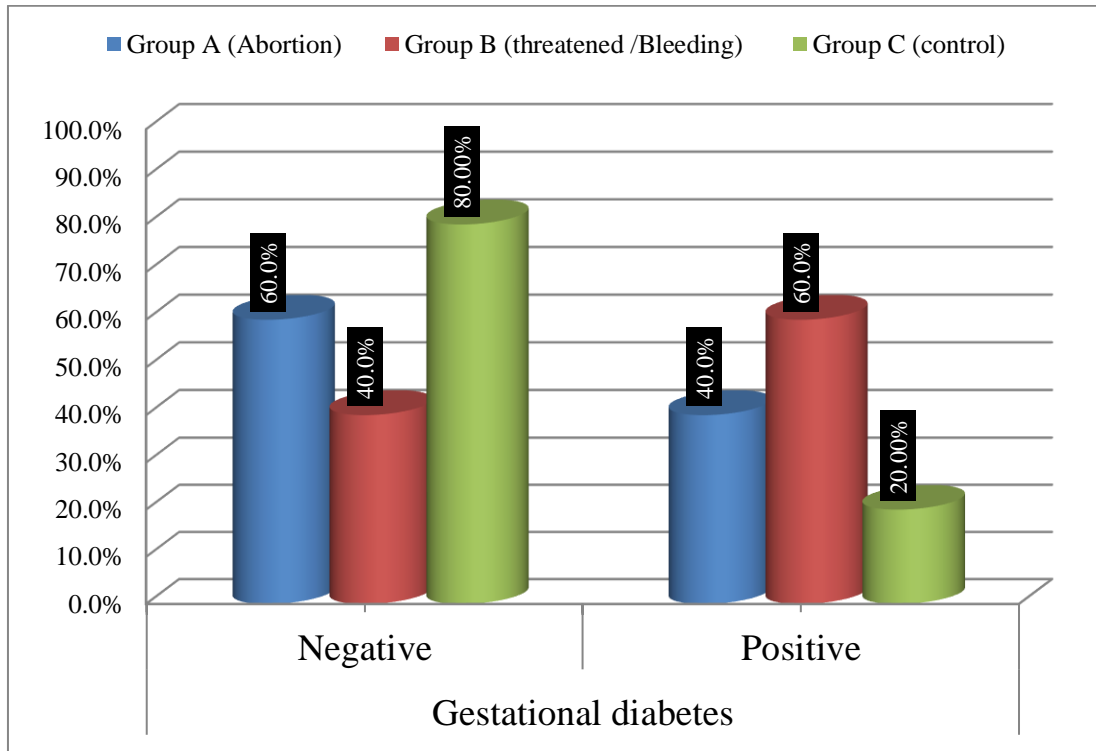

Figure 5. Comparison between all the studied groups according to
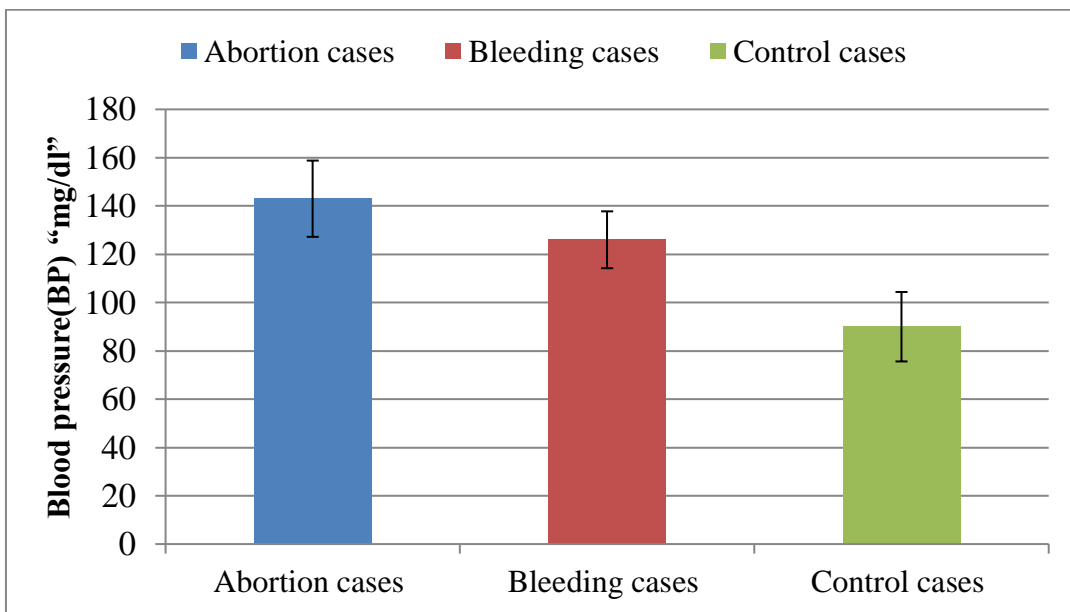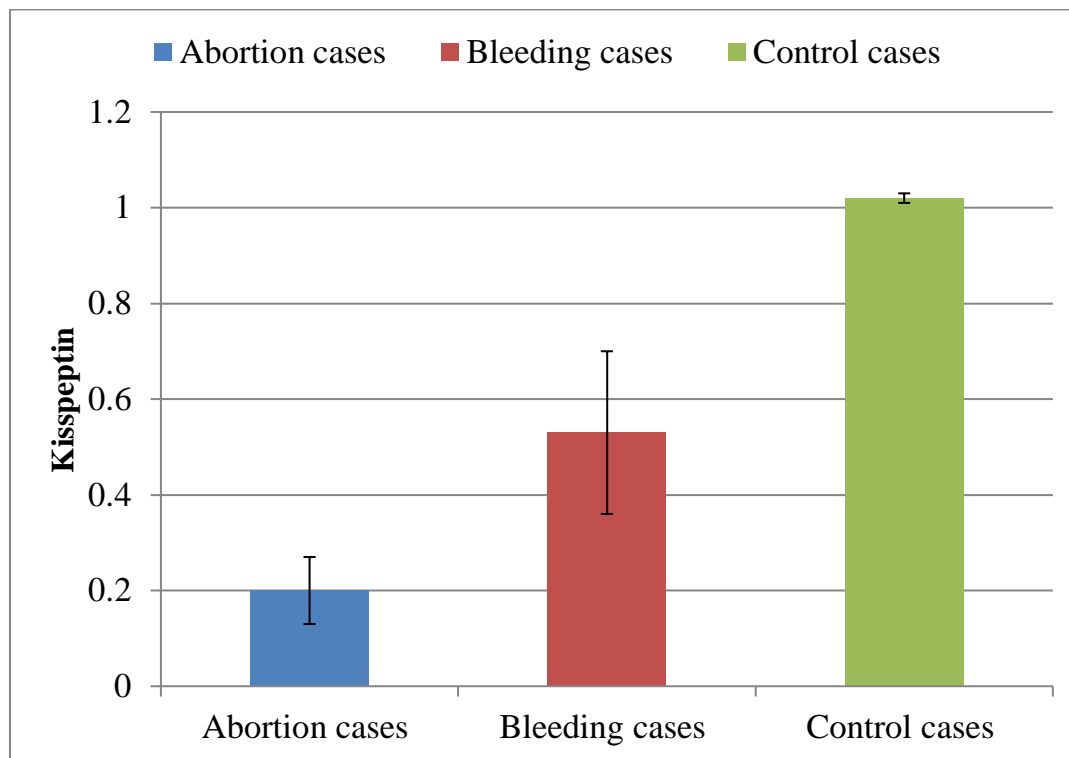blood pressure "mg/dl"

Figure 6. Comparison between all the studied groups according to Kisspeptin

Discussion

Spontaneous abortion refers to the phenomenon by which an embryo or fetus is discharged automatically from the mother's body for some reason, usually at 28 weeks of pregnancy [17]. As regard demographic data , our study showed a highly statistically significant difference between all the studied groups as regard body mass index, with Mean±SD (28.90±3.54 Kg/m2), Range (22-35) for group A , Mean±SD (28.00±3.40 Kg/m2), Range (23-34) for group B and Mean±SD (21.10±2.25 Kg/m2), Range (18-25) for group C and with p-value (p<0.001) for all the studied groups as represented by figure (1) . this result is in agreement with metwlly et al [18] who reported that increase body mass index is associated with an increased risk of first trimester miscarriage and women with BMI >25 kg/m2 had significantly higher risk of miscarriage . our study showed that there was no statistically significant difference between all the studied groups as regard Maternal age with Mean±SD(34.60±6.69), Range (24-44) for group A , Mean±SD (34.40±6.54), Range (23-43) for group B and Mean±SD (31.50±4.74), Range (26-40) for group C and with p-value (0.414) as represented by figure (2). This was in agreement with Dadkhah et al [19] who did not have any significant differences as regard maternal age in women with advanced maternal age on evaluation of the pregnancy outcome in cases with threatened miscarriage. However, these results are not in agreement with Mbugua

et al [20] who reported that advancing maternal age is associated with an increased risk of miscarriage. also we found no statistically significant difference between all the studied groups as regard Gestational age with Mean±SD(9.20±1.69), Range (7-12) for group A , Mean±SD (8.80±1.03), Range (8-10) for group B and Mean±SD (8.40±1.07), Range (7-10) for group C and with p-value (0.368) as represented by figure (3). While Gestational diabetes mellitus (GDM) was described by positive or negative which was represented by percentage (%) where (60.0%) of group A was negative and (80.0%) of group C was negative but (60.0%) of group B was positive at p-value (0.174).This was represented by figure (4). however, Calleja-Agius et al [21] reported that no relationship exists between threatened miscarriage and GDM.

Also we found a highly statistically significant difference between all the studied groups according to mean arterial blood pressure (MAP), with Mean±SD (143.00±15.7mg/dl), Range (120-170) for group A, Mean±SD (126.00±11.7mg/dl), Range (105-145) for group B and Mean±SD (90.00±14.3 mg/dl), Range (70-110) for group C and with p-value (p<0.001) for all the studied groups as represented by figure (5). This was in agreement with Davey DA et al [22] who stated that Hypertension during pregnancy is not only common but also associated with a risk of morbidity and mortality. as regard relative expression of kisspeptin we found that there

was highly statistically significant higher mean value of Kisspeptin in control group was 1.02±0.01, Range (1-1.04) followed by Bleeding Cases was 0.53±0.17, Range (0.27-0.79) and the lowest mean value in Abortion Cases was 0.20±0.07, Range (0.11-0.33) and with p-value (p<0.001) for all the studied groups as represented by figure (6). BILBAN M et al [23] reported that the placenta is believed to be the source of increased expression of kisspeptin in normal pregnancy cases, because it returns to non-pregnant levels immediately after birth. our results are in agreement with Jayasena CN et al who demonstrated that the hormone levels of kisspeptin in patients who had a miscarriage were lower compared with those in controls [24].however our results are not in agreement with NIJHER et al who didn't find any significant difference in circulating KP-10 levels between patients with preeclampsia (PE) complicated with intrauterine growth retardation and normotensive controls in late pregnancy [25]. Horikoshi et al, reported for the first time that the plasma concentration of KP increased dramatically throughout the gestation, elevating to 1230 fmol/mL in the first trimester and reaching a maximum level of 9590 fmol/mL in the third trimester. KP levels then returned to 7.6 fmol/mL by postpartum day 5 and the peripheral KP levels were found to be very low and did not increase during pregnancy in sheep, cows, pigs, rabbits, horses, rhesus monkeys and marmosets, suggesting that the increase in plasma KP levels during pregnancy is unique to humans [26]. However our results are not in agreement with Grokem et al, who concluded that there was no relationship between serum kisspeptin concentrations and pregnancy outcomes in the first trimester of pregnancy [27].

Conclusion

The results of our study strongly suggest a significant association between spontaneous abortion risks and lower circulating Kisspeptin concentrations in early pregnancy. Kisspeptin appears as a new biomarker for the early detection of spontaneous abortion. These findings set the stage for further biomarker validation in larger randomized controlled trials.

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