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Assessment of steroidal hormones levels from serum and saliva in females

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Dedication

To … My wounded country

Patient and wise man My father

Lovely and kindness woman My mother

Compassionate and donate ideal My aunts

My dear sisters and brother

My all friends

And who help me……...

Noor adil

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Conclusions

1. Salivary hormonal test is a useful method for assessment progesterone and cortisol, while it is not useful test for measuring salivary estradiol.
2. Mean levels of salivary steroidal hormones were reduced as compared to serum levels.
3. Strong correlation appeared between serum and salivary progesterone, and salivary cortisol, and there was no correlation between serum and salivary estradiol.

Suggestions

1. Evaluation of salivary steroidal hormones levels in serum and saliva of large numbers of healthy and infertile females.
2. Assessment and comparison of steroidal hormones levels from serum and saliva in healthy males.

Discussion

The period of menstrual cycle for females, included in this study ranged (22-33) days, this represents the normal menstrual range for healthy females according to results mentioned by Mishell (2001) and Speroff and Fritz (2005). It was known that the period of menstrual cycle is affected by several factors including FSH and LH (Ganong, 2003), GnRH (Knobil, 1988), temperature (Rojansky *et al*., 1992) and stress (Davydov *et al*., 2007). The gonadotropin releasing hormones (GnRH) released from hypothalamus regulates the pituitary secretion of gonadotropins (FSH and LH) that act on ovaries and therefore ovarian hormones change during menstrual cycle (Mayne, 1994).

From the results of the present study for infertile females, it appeared that the level of serum progesterone for infertile female with secondary infertility reduced significantly ($P<0.05$) as compared with primary infertility females. Similar results were reported for level of salivary progesterone. It was reported that the most infertile females with secondary infertility complaining from abnormal ovarian functions (Potter, 2006), while primary infertility females had complaining from severe damage in the reproductive system, and with less degree abnormal ovarian function (Aljabri, 2005).

Clearly, in the present study, the result for levels of serum and salivary progesterone were well positively synchronized as in elevation and vice versa. This result was in agreement with other studies (Meulenberg and Hofman, 1989; Vuorento *et al*., 1989). Therefore, assessment of the level of salivary progesterone may be act on in clinical evaluation of females in addition to academic research (Choe *et al*., 1983; Delfs *et al*., 1994; Harris *et al*., 1994).

In general, levels of progesterone in saliva were less than serum as noticed from the result of this study. Similar studies reported same results (Wang and knyba, 1985; Swinkels *et al*., 1990). Within saliva, only free

steroidal hormones were evaluated which constitute about 5-8% of total steroidal level (Ferguson *et al*., 1984). Large amount of steroids 92-95% in the blood are bound by proteins like sex hormone binding globulin (SHBG), cortisol binding globulin (CBG), and albumin (Vittek *et al*., 1985), which make the steroid molecules large and cannot enter the capillary beds of tissues (Koefoed and Brahm, 1994; Burry *et al*., 1999).

Although, assessment of serum estradiol observed high levels, while assessment the level of salivary estradiol was not reproducible, or not sensitive, as presented in this study. Different factors may explain this results including short period for peak of estradiol level (Lee *et al*., 2004; Guballa *et al*., 2007). Also, low percentage of free estradiol which constitute about no more 5% of total estradiol (Worthman *et al*., 1990). Furthermore the presence of free chemical structure of estrogen involving estradiol, estriol, and estron may be affect final result within saliva (Behl *et al*., 1995). However, other studies reported that the assessment of salivary estradiol is possible (Mounib *et al*., 1988; Deboever *et al*., 1990).

Assessment of serum cortisol level were determined in this study which showed that level of serum cortisol reduced significantly from females with primary infertility to females with secondary infertility, this result correspond with other study (Wilson *et al*., 1998).

Results for levels of salivary cortisol appeared that there was synchronization with level of serum cortisol either in elevation or reduction, this result agreed with Aardal-Eriksson *et al*. (1998), which they stated that level of serum cortisol was synchronized as elevation and reduction with level of salivary cortisol. Our study showed that almost specimen of salivary cortisol not sensitive, this may be belong to females are under stress from vienpuncture that affect the result (Vining *et al*., 1983; Khan-Dawood *et al*., 1984; Kiess *et al*., 1995; Findling and Raff, 2001).

Females ages ranged from (16-55), with mean age was (35.5 ± 1.8 years old) as presented in table (1), which is considered to be an important

period in which the conception rate begins to decline significantly due to neuroendocrine changes that end with state of menopause (Burger *et al*., 1999).

In this study, it was found that the highest levels of serum progesterone were within age group 30-39 years old, while the lowest level of progesterone were within age group ≥50 years. This result agreed with study of Cramer *et al*. (2002), which proposed that there is an increasing level of reproductive hormones within age of 30-40 years old. A significant reduction in salivary progesterone as the age of females in the present study increases this result agreed with Lipson and Ellison (1992). Several studies appeared that level of progesterone decline as the age of females increase combined with degradation of follicles (Meldrum *et al*., 1981; Snowdon, 1990; Hirshfield, 1991).

The level of serum estradiol in group 30-39 years old was more than other groups; this increase may be due to loss of ovarian feed back on the hypothalamus and pituitary component of the reproductive axis (Lee *et al.*, 1988). However, level of estradiol decreased as female age increase, this is due to reduction in follicle number with increasing age, therefore low levels of estradiol is produced and consequently affect LH level through positive feed-back of hypothalamus (Lenton *et* al., 1984; Nedelikovic and salvic, 1990; Scheffer *et al*., 1999).

From the results of this study it was shown that there was a significant ($P<0.05$) reduction in the level of serum and salivary cortisol as the age of females increase, and this explained the presence of synchronization between salivary cortisol and serum. Several studies appeared that stress causes elevation level of cortisol, since younger females exposed to life challenging and difficulties in work more than older one, this causes stress and increasing level of cortisol (Kirschbaum *et al*., 1999; Al'Apsi *et al*., 1997; Vedhara *et al*., 1999).

According to levels of hormones within serum and saliva for single and married females, significant ($P<0.05$) reductions were assessed for progesterone, estradiol and cortisol (Table 6). Rodriguez *et al*. (2001) reported that reduction in the level of steroidal hormones was observed between serum to saliva. The cause for this reduction is due to presence of free steroidal hormones in little amount within saliva than in serum.

Although, the mean level of serum progesterone for married females was more than the mean level of serum progesterone for single females, but did not reach the level of significance ($P<0.05$). Same results were reported for estradiol and cortisol. These results were in agreement with other research was reported by Cai *et al*. (1993). It was known that level of steroidal hormones for married impregnate females have similar result for single females in assessment of steroidal hormones levels (Gann *et al*., 2001; Lobo *et al*., 2003).

Significant ($P<0.05$) differences were reported for levels of three steroidal hormones in both serum and saliva of fertile and infertile females in this study. These results may be explained that the cause of infertility for females is not related to disturbances in the hormonal balance of hypothalamus-pituitary ovary axis. It was noticed that several causes of female infertility resulted from disturbances and/or anomalies in the female reproductive ducts (Parr and McMahon, 1998). Similar results were certified that these abnormalities have major causes of female infertility (Kooke, 1999), also, most endocrinological causes of female infertility were easier to be overcome through hormonal treatment (Anthony *et al*., 2001).

Hormonal results for females, in this study, were classified according to marital and fertility status appeared non significant ($P>0.05$) differences in the level of salivary steroidal hormones. While significant ($P<0.05$) differences were reported in the hormonal levels of serum females among the three groups. This combination in the marital and fertility status results are agreement with several studies (Li *et al*., 1989; Kalmuss, 1987).

It was reported that the disturbances in some females results from marriage (Mishell, 2001), and/or uses of different hormonal treatments (Kousta *et al*., 1997). However, the fertility status of females has wide range of steroidal hormonal levels and affected with age of marriages (Menken *et al.*, 1986). Other factors are effects the fertility of either married or single females including stress (Hall *et al*., 1985), hormonal balance (Carr *et al*., 1998), age (Forero *et al*., 1987), number of previous pregnancy (Mittendorf *et al*., 1990), body weight (Perego *et al*., 2005), body mass index (Winnicki *et al*., 2002), in addition social and psychological factors (Larsen *et al*., 2003).

However, Lipson and Ellison (1989) revealed advantage of salivary hormone testing is that it is easy, as well as more convenient for the patient, saliva can be easily obtained at desired times without disrupting laboratory schedules for phlebotomy. Furthermore, since saliva collection is typically performed at home or in the workplace, there is high patient compliance (Read, 1993; Pugeat *et al*., 1996). For women who work in an occupational setting, followed by 24 to 40 hours per week of tasks related to childcare, household duties, community and/or volunteer work, being required to go to a laboratory 1 to 4 times during a day or at specific phases of her menstrual cycle may well be impractical and inconvenient (Petsos *et al*., 1986).

Another advantage of salivary testing is cost; the cost of salivary hormone analysis is markedly less than that of serum testing. For example, a single hormone test for salivary progesterone averages less than one-fourth the cost of a single serum level (Bakerman, 2002). Considering that patients usually undergo evaluation of more than one hormone, such cost differences can be pronounced (Riad-Fahmy *et al*., 1983).

Introduction

Hormones are secreted within endocrine glands and from there they move into other extracellular spaces. Several hormones are distributed widely following secretion and equilibrate throughout virtually all extra-cellular spaces, including formed saliva stored in the salivary glands (Nabipour, 2003). Hormones act over a range of distances and many of their functions are integrative and are aimed at co-coordinating the wide variety of functions required to maintain optimal overall wellness (Worthman *et al*., 1990). They can enter saliva by a variety of mechanisms but for the neutral steroids the most common route is rapid diffusion through the acinar cells and their concentration is independent on the rate of saliva flow (Vining *et al*., 1983).

As hormones play such an important role in the management of health, the determination of a hormonal imbalance can help identify the cause of many health problems (Lu *et al*., 2006). Addressing these imbalances with appropriate testing and treatment protocols provide a direct basis for maintaining optimal health and wellbeing (Kirschbaum and Hellhammer, 1994). Using radioimmunoassay techniques that are suitable for measuring the low concentrations of steroid hormones in saliva (Vittek *et al*., 1985)

Salivary hormone analysis is a useful clinical tool in the assessment of hormones in females and scientific evidence supports its use because it offers an accurate and comprehensive reflection of some of hormone bioavailability at the tissue level values for salivary hormone levels have been established for females (Riad-Fahmy *et al*., 1983). As biological medium saliva is a variable and complex fluid and is mostly produced by three pairs of salivary glands (parotid, submandibular and sublingual) with a small contribution from the buccal glands which line the mouth (Ferguson, 1984).

Lo *et al*. (1992) revealed that some patients are significantly stressed when undergoing phlebotomy, and changes induced by vein puncture stress can significantly affect serum values. Thus, the serum value may not be an accurate measure of the patient's usual endogenous hormone level. In contrast, saliva testing has not been shown to cause a stress response in patients (Swinkels *et al*., 1992). Therefore the aims of this study are:

1. Assessment of serum and salivary steroidal hormones (progesterone, estradiol and cortisol) in females classified according to age groups, type of infertility, marital status, and fertility status.
2. Evaluation the synchronization in the level of steroidal hormones between serum and saliva of females.
3. To determine if saliva as diagnostic tool for measurement of steroidal hormones in females.
4. To determine the effectiveness of salivary hormone testing advantage rather than serum measurements.

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Abbreviations

ACTH	Adrenocorticotropic hormone
AFs	Activation function domains
AF3	Activation function domain 3
AP-1	Activator protein 1
CBG	Cortisol binding globulin
CRH	Corticotropin-releasing hormone
DNA	Deoxyribonucleic acid
E_2	Estradiol
ERα	Estradiol receptor α
ERβ	Estradiol receptor β
FSH	Follicle stimulating hormone
GnRH	Gonadotropine releasing hormone
GR	Glucocorticol receptor
Hsp90	Heat shock protein 90
Hsp70	Heat shock protein 70
LH	Luteinizing hormone
M1	First Meiotic division
mRNA	messenger Ribonucleic acid
NF-$_K$B	Nuclear factor-kappa B
PR-A	Progesterone receptor A
PR-B	Progesterone receptor B
RIA	Radioimmunoassay
SERMs	Selective estrogen receptor modulators
SHBG	Sex hormone binding globulin

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Results

In the present study, ninety females were enrolled, and their ages were ranged from (16-55) years with period for menstrual cycle ranged (22-33) days (Table 3-1). From the same table, duration of infertility for infertile females was (2-17) years. Within infertile females, the percentage of infertile female with primary (1°) infertility and secondary (2°) infertility were 62.96% and 37.03% respectively (Table 3-1).

Figure (3-١) shows the numbers of females were classified according to age groups. This figure showed the normal distribution curve of statistical distribution. However, the largest age group of females was occupied by the age group (30-39) years. In contrast, the age group (≥ 50) years was appearing the smallest group of females in the present study.

The highest level of serum progesterone for females was observed within age group (30-39 years). While, the lowest level of serum progesterone was assessed for females of the age group (≥ 50 years) as presented in the table (3-٢). From the same table, results of serum E_2 for females with age group (30-39 years) appeared highest level of serum E_2. Conversely, the lowest level of E_2 was evaluated for females within age group (20-29 years). According to the results of serum cortisol, gradual reduction in the level of cortisol as age of female increased (Table 3-٢).

The levels of salivary progesterone were reduced as age of female increased (Table 3-2). Same results were evaluated for level of salivary E_2 for all age groups of females as presented in the table (3-2). From same table, little differences in the levels of salivary cortisol were reported among different age groups.

Table 3-1: Descriptive parameters of females enrolled in the present study.

Parameter	Range
Age (years)	16-55 (mean: ٣٥,٥±1.8)
Menstrual cycle period (days)	22-33 (mean: ٢7.5±0.9)
Duration of infertility (years)	2 -17 (mean: ٩,٥±1.1)
Ratio of infertile females with 1º infertility (%) **	34/54 (62.96)
Ratio of infertile females with 2º infertility (%) ***	20/54 (37.03)

- Data are Mean ± SE
- ** Number of infertile female with primary infertility (1º) =34
- *** Number of infertile female with secondary infertility (2º) =20

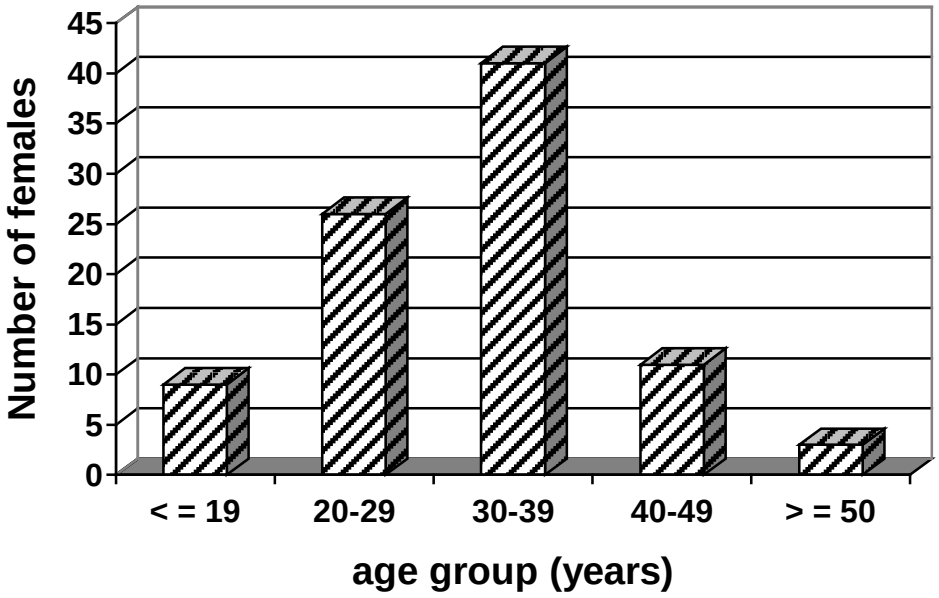

Figure ٣-١: Number of females classified according to age groups*

Data= Mean ± SE

*** Mean age for females (35.5 ± 1.8 years)**

Table 3-٢: Serum and salivary levels of steroidal hormones for females enrolled in the present study classified according to age group*

Age group (years)	Progesterone (ng/mL.)		Estradiol (pg/mL.)		Cortisol (µg/dL.)	
	serum	saliva	serum	saliva	serum	saliva
≤19 (No.=9)	١,٧٣١ ± ٠,٣٧٧٣	٠.090 ± 0.026	89.49 ± 30.046	6.000 ± 0.000	20.804 ± 7.896	0.360 ± 0.00
20-29 (No.=26)	3.251 ± 0.914	0.074 ± .0115	68.35 ± 8.686	6.000 ± 0.000	17.316 ± 1.195	٠.351 ±0.351
30-39 (No.=41)	3.503 ± ٠.1261	0.066 ± 0.0111	192.07 ± 67.19	6.000 ± 0.000	14.477 ± 0.833	0.360 ± 0.00
40-49 (No.=11)	2.824 ± 2.591	0.064 ± 0.0056	79.29 ± 24.27	6.000 ± 0.000	13.678 ± 1.305	0.360 ± 0.00
≥50 (No.=3)	1.373 ± 0.173	0.050 ± 0.0000	92.57 ± 16.83	6.000 ± 0.000	8.926 ± 1.248	0.360 ± 0.00

- Data= Mean ± SE.
- * Mean age for females (35.5 ± 1.8 years)

Significant (P<0.05) differences in the level of progesterone were obtained between serum and saliva among different female age groups (Figure 3-2). Similar results were reported for E_2 (Figure 3-3) and cortisol (Figure 3-4). From the results of the present study, it was appeared that serum progesterone level has positive correlations and significant with salivary progesterone within age group ≤19 (r=0.725; P=0.02), positive correlations and highly significant within age group 20-29 years (r=0.754; P=0.001), and for age group 40-49 years the result was positive correlations and significant (r=0.649; P=0.031).

According to type of infertility, significant (P<0.05) reduction was noticed in the mean level of the serum progesterone for infertile females with 2° infertility as compared to infertile females with 1° infertility (Table 3-3). Also significant (P<0.05) reduction was assessed in the serum level of estradiol (E_2) for infertile females with 1° infertility when compared to 2° infertility. Level of serum cortisol was reduced significantly (P<0.05) for infertile female with 2° infertility as compared to female with 1° infertility (Table 3-3).

The results of steroidal hormones in saliva of infertile females classified according to type of infertility were presented in the table (3-4). Non-significant (P>0.05) differences were assessed for levels of progesterone, E_2 and cortisol between infertile females with 1° infertility compared to with 2° infertility. Significant (P<0.05) reduction was observed in the mean level of salivary progesterone as compared to serum level of progesterone for both types of infertility (Figure 3-٥). Similar results were reported for E_2 (Figure 3-٦) and cortisol (Figure 3-٧).

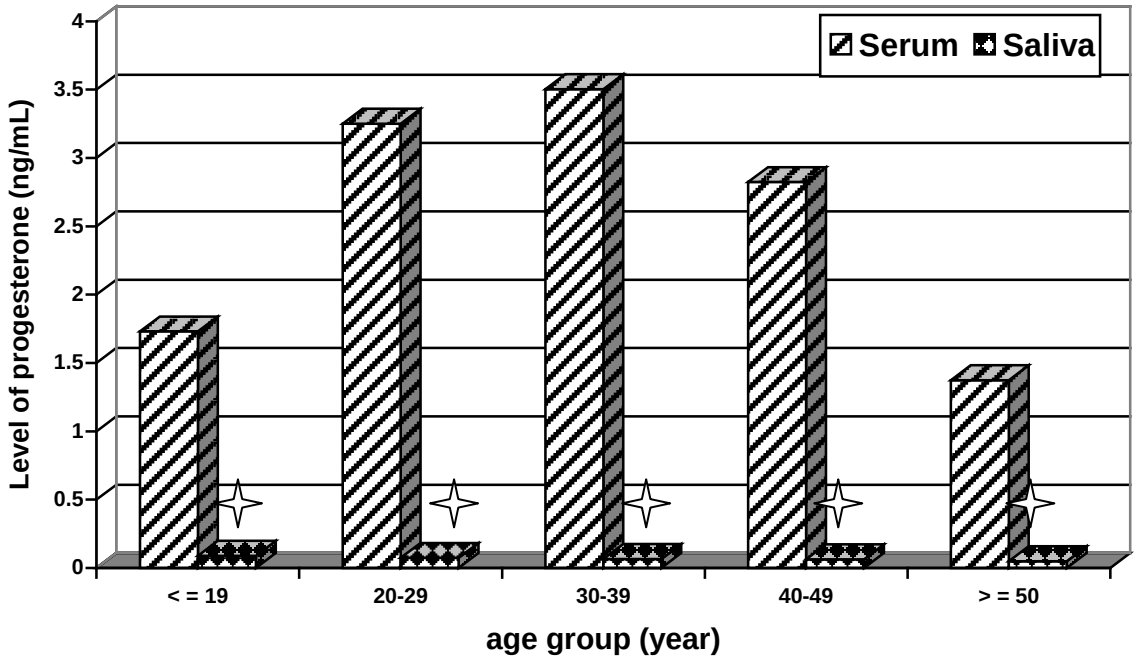

Figure 3-٢: Mean level of serum and salivary progesterone for females classified according to age groups

✧ **Significant (P<0.05) reduction as compared to it corresponding group.**

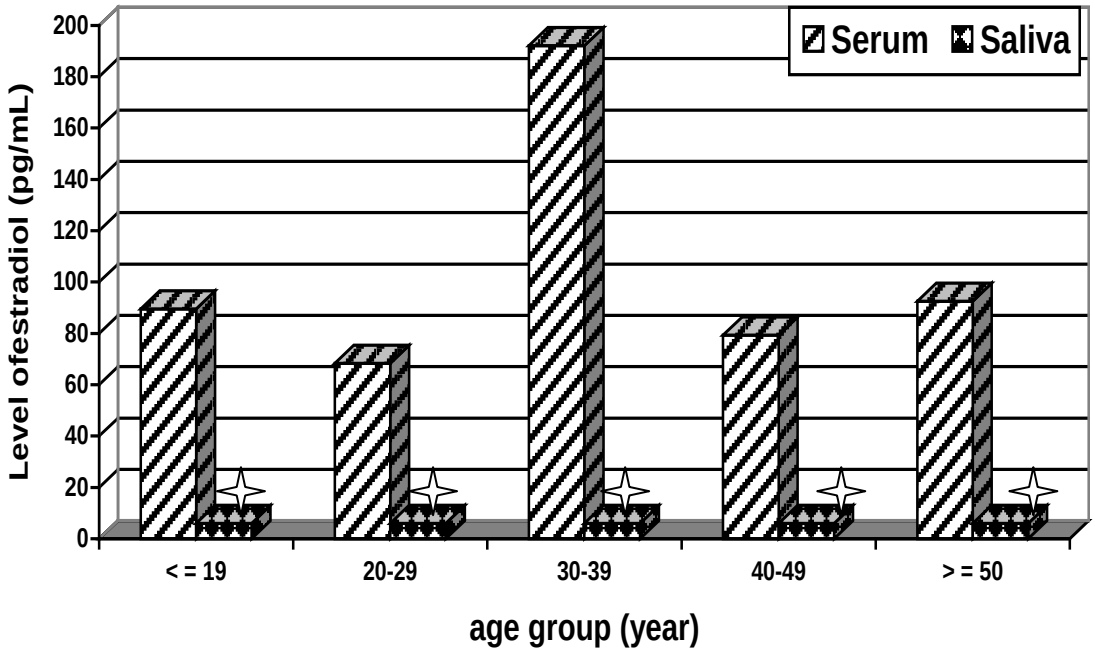

Figure ٣-٣: Mean level of serum and salivary estradiol for females classified according to age groups

✧ **Significant (P<0.05) reduction as compared to it corresponding group.**

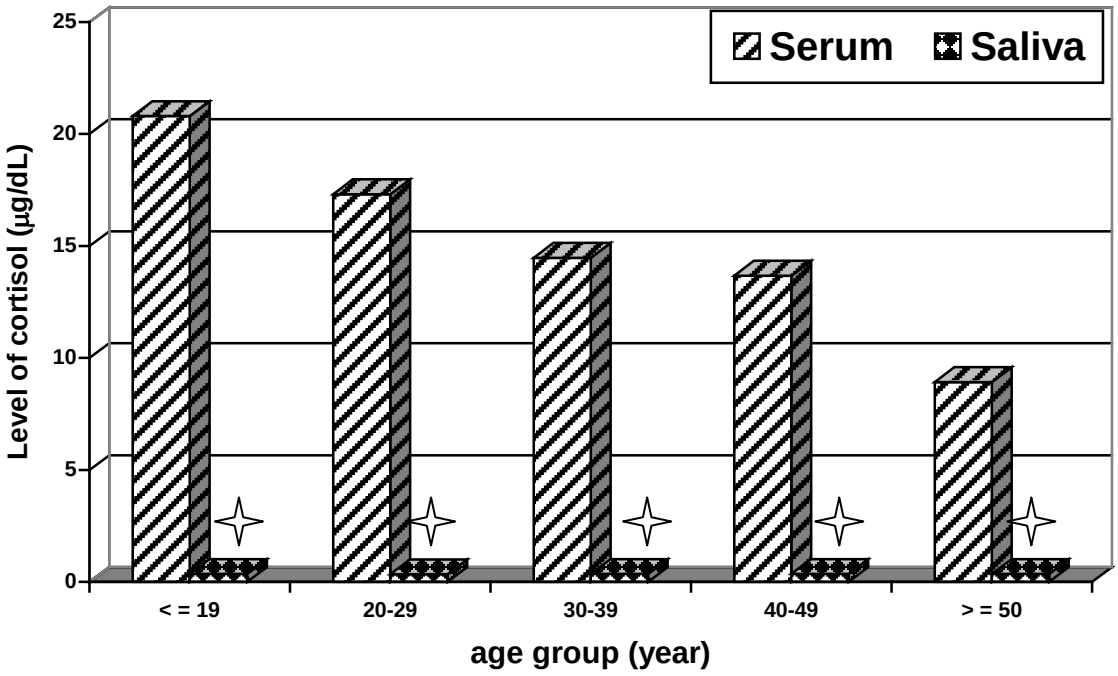

Figure 3-٤: Mean level of serum and salivary cortisol for females classified according to age groups

✧ **Significant (P<0.05) reduction as compared to it corresponding group**.

Table 3-3: levels of serum steroidal hormones for infertile females classified according to type of infertility

Levels of serum hormones	Type of infertility	
	1° infertility (No.=34)	2° infertility (No.=20)
Progesterone (ng/mL.)	4.364 ✧ ± ١,٠٤١	2.399 ± ١,٢٢٨
Estradiol (pg/mL.)	75.56 ✧ ± ٧,٧٤٨	105.8 ± ١٢,٠٥
Cortisol (µg/dL.)	19.14 ✧ ± ٢,٣٢٦	12.39 ± 0.600

- Data are Mean ± SE.
- ✧ Significant (P<0.05) differences for serum progesterone between primary and secondary infertility.
- ✧ Significant (P<0.05) differences for serum estradiol between primary and secondary infertility.
- ✧ Significant (P<0.05) differences for serum cortisol between primary and secondary infertility.

Table 3-4: levels of salivary steroidal hormones for infertile females classified according to type of infertility

Levels of saliva hormones	Type of infertility	
	1° infertility (No.=34)	2° infertility (No.=20)
Progesterone (ng/mL.)	0.074 ✧ ± ٠,٠٠٩	٠,٠٦١ ± 0.0059
Estradiol (pg/mL.)	6.000 ✧ ± 0.00	6.000 ± 0.00
Cortisol (µg/dL.)	0.358 ✧ ± ٠,٠٠٣	0.360 ± 0 .00

- Data are Mean ± SE.
- ✧ Significant (P<0.05) differences for salivary progesterone between primary and secondary infertility.
- ✧ Significant (P<0.05) differences for salivary Estradiol between primary and secondary infertility.
- ✧ Significant (P<0.05) differences for salivary cortisol between primary and secondary infertility.

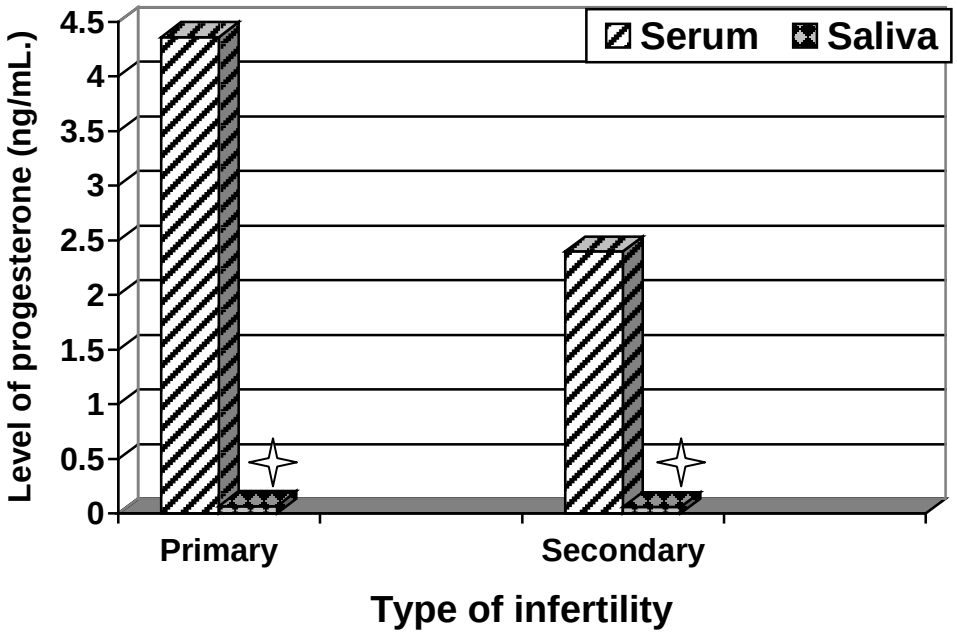

Figure 3-٥: Mean levels of serum and salivary progesterone for infertile females classified according to type of infertility

Data are Mean ± SE.

✧ **Significant (P<0.05) differences between serum and salivary progesterone for primary and secondary infertility.**

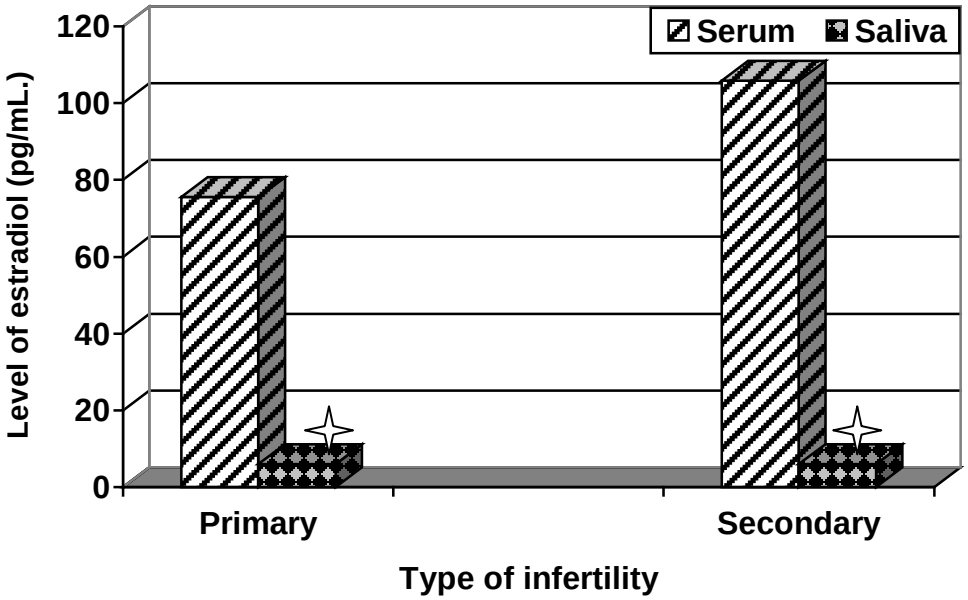

Figure 3-٦: Mean levels of serum and salivary estradiol for infertile female classified according to type of infertility

Data are Mean ± SE.

Significant (P<0.05) differences between serum and salivary estradiol for primary and secondary infertility.

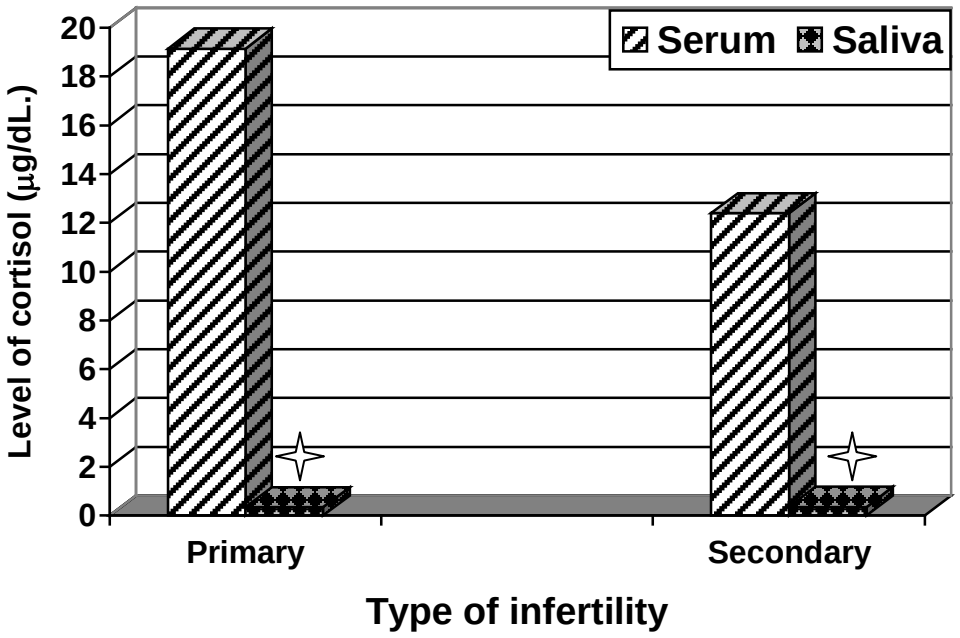

Figure 3-٧: Mean levels of serum and salivary cortisol for infertile females classified according to type of infertility

Data are Mean ± SE.

✧ **Significant (P<0.05) differences between serum and salivary cortisol for primary and secondary infertility.**

For females with 1° infertility, it was appeared a positive correlation and highly significant (r=0.603; P=0.001) were evaluated between serum and salivary progesterone. However, significant correlation was assessed between serum and salivary cortisol (r=0.485; P=0.023).

Statistical assessment of hormones between serum and saliva for both single and married females were noticed in table (3-5) that shows significant (P<0.05) differences in the progesterone, E_2 and cortisol levels between serum and saliva for single female. Highly significant (P<0.01) differences in the level of progesterone was observed between serum and saliva for married females. From same table, levels of E_2 and cortisol showed highly significant (P<0.01) differences between serum and saliva for both single and married females.

Non significant (P>0.05) differences in the level of serum and salivary progesterone were observed between single and married females (Figure 3-8). Similarly, E_2 and cortisol levels in serum and saliva showed non significant (P>0.05) differences between single and married females (Figures 3-9 and 3-10; respectively). It was observed a positive correlation and highly significant between serum and salivary progesterone for married infertile females (r=0.566; P=0.001), also for single infertile females, highly positive and significant correlation (r=0.869; P=0.001). On the other hand, non significant correlation between serum and salivary cortisol was appeared.

In the table (3-6), females classified according to fertility status into fertile and infertile, however there are significant (P<0.05) differences were assessed between serum and salivary progesterone for fertile females and also for infertile females. From same table, it was noticed highly significant (P<0.01) differences in E_2 level for serum and saliva between fertile and infertile females. Same results were observed for serum and salivary cortisol between fertile and infertile females.

Table 3-5: Levels of serum and salivary steroidal hormones for females classified according to marital status

Levels of hormones	Single females (N0. =20)		Married females (N0. =70)	
	Serum	Saliva	Serum	Saliva
Progesterone (ng/mL)	2.356 ✧ ± ٠,٨٦٦	0.081 ± ٠,٠١٤	3.253 ✧ ± ٠,٨١٢	0.066 ± ٠,٠٠٥
Estradiol (pg/mL)	59.17 ✧✧ ± ٥,٨١٧	6.00 ± ٠,٠٠	91.17 ✧✧ ± ١١,٣٨	6.00 ± 0.00
Cortisol (µg/dL)	16.48 ✧✧ ± ١,١٨٣	0.353 ± ٠,٠٠٦	15.41 ✧✧ ± 1.159	0.358 ± ٠,٠٠١

- Data are Mean ± SE.
- ✧ Significant (P<0.05) differences between serum and saliva
- ✧✧ High significant (P<0.001) differences between serum and saliva

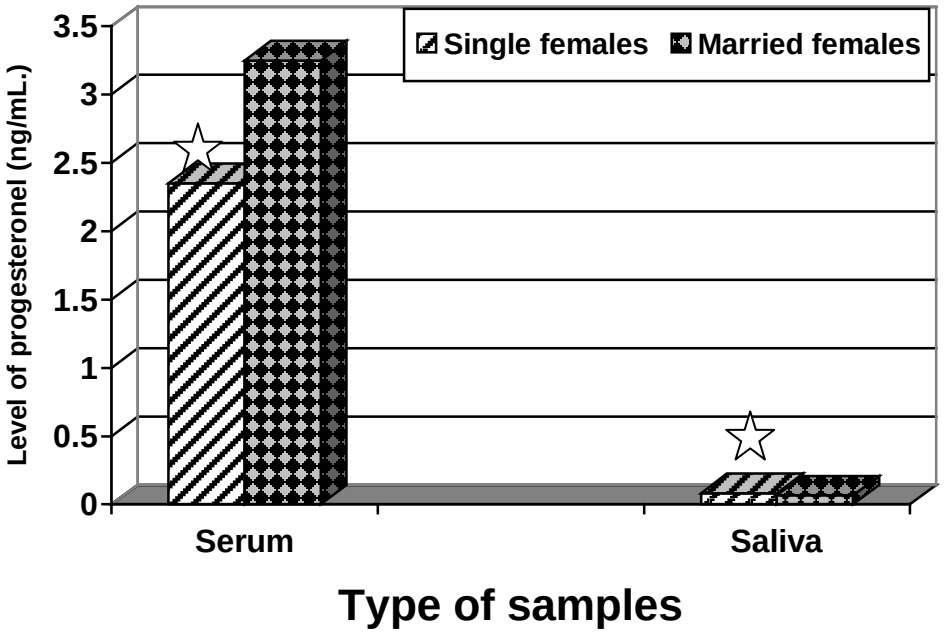

Figure 3-8: Mean level of serum and salivary progesterone for single and married females

Data are Mean ± SE.
☆ **Non significant (P>0.05) differences for serum and salivary progesterone between single and married females**

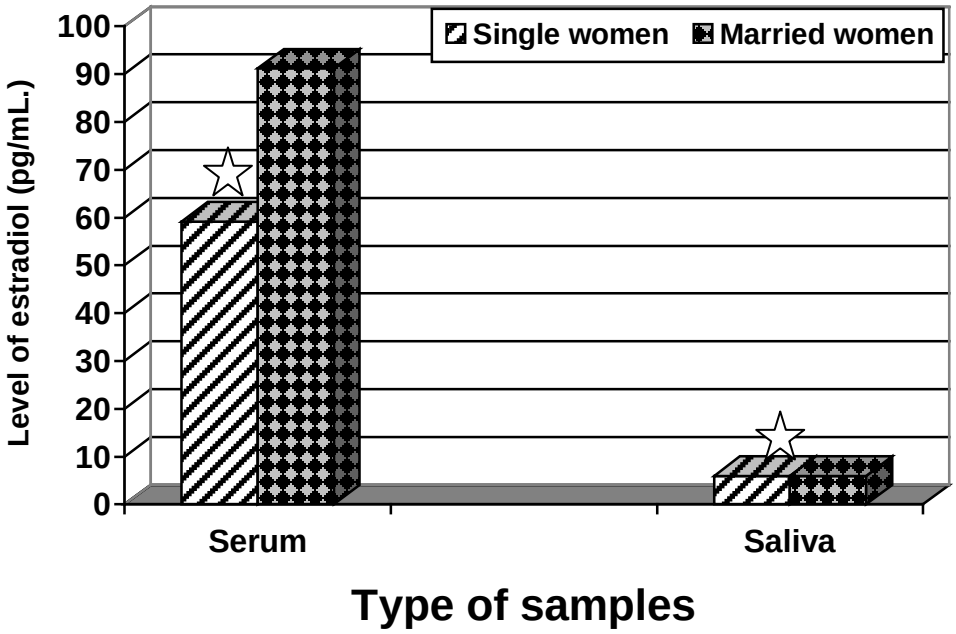

Figure 3-9: Mean level of serum and salivary estradiol for single and married females

Data are Mean ± SE.

☆ **Non significant (P>0.05) differences for serum and salivary estradiol between single and married females.**

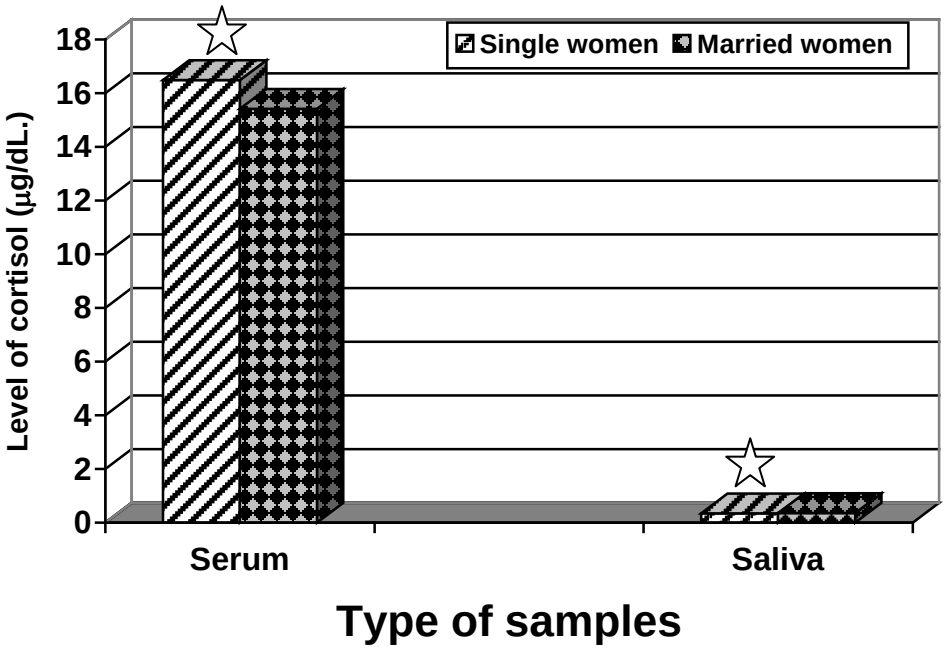

Figure 3-10: Mean level of serum and salivary cortisol for single and married females

☆ **Data are Mean ± SE.**
Non significant (P>0.05) differences for serum and salivary cortisol between single and married females.

Table 3-6: Levels of serum and salivary steroidal hormones for married females classified according to fertility status

Levels of hormones	Fertile females (No. =16)		Infertile females (No. =74)	
	Serum	Saliva	Serum	Saliva
Progesterone (ng/mL.)	3.325 ✧ ± 1.187	0.071 ± 0.0081	3.288 ✧ ± 0.709	0.074 ± 0. 007
Estradiol (pg/mL.)	108.88 ✧✧ ± 18.27	6.000 ± 0.000	63.85 ✧✧ ± 4.396	6.00 ± 0.00
Cortisol (µg/dL.)	13.24 ✧✧ ± 0.623	0.36 ± 0.000	17.75 ✧✧ ± 1.546	0.355 ± 0.003

- Data are Mean ± SE.
- ✧ Significant (P<0.05) differences between serum and saliva.
- ✧✧ High significant (P<0.001) differences between serum and saliva.

Results of serum and salivary progesterone appeared non significant (P>0.05) differences between fertile and infertile females (Figure 3-11). While, levels of serum E_2 and cortisol were differed significantly (P<0.05) between fertile and infertile females (Figures 3-12 and 3-13; respectively). From same figures, salivary estradiol and cortisol appeared non significant (P>0.05) differences between fertile and infertile females.

The result of correlation test appeared that there was a positive correlation and significant between serum and salivary progesterone (r=0.298; P=0.05) for fertile females. For infertile females, positive correlation and highly significant was appeared between serum and salivary progesterone (r=0.612; P=0.001). However, non-significant correlation was noticed between serum and salivary cortisol for infertile female. The results of steroidal hormones in the serum and saliva of females were classified according to marital and fertility status presented in the table (3-7). Significant (P<0.05) reduction was observed in the level of salivary progesterone as compared to serum progesterone of married fertile females. Similar results were reported for married infertile and single infertile females (Table 3-7).

From same table, level of salivary E_2 and cortisol were significantly (P<0.05) reduced when compared to levels of serum E_2 and cortisol for three females groups. A clear synchronization was noticed between levels of progesterone and cortisol in serum and saliva for all groups of females, in contrast to estradiol (Table 3-7).

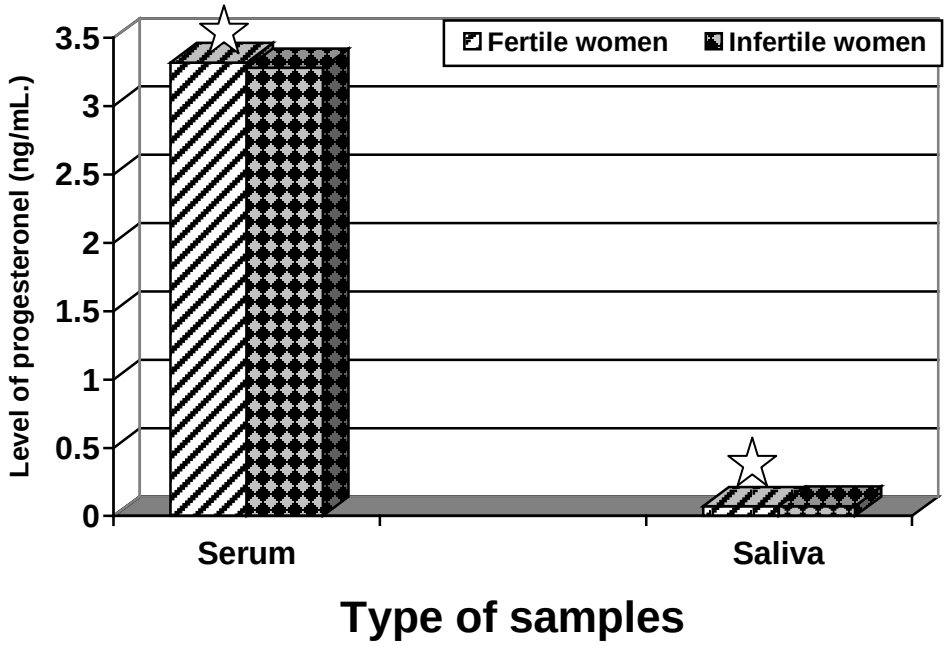

Figure 3-11: Mean level of serum and salivary progesterone for fertile and infertile female

Data are Mean ± SE

☆ **Non significant (P>0.05) differences for serum and salivary progesterone between fertile and infertile females.**

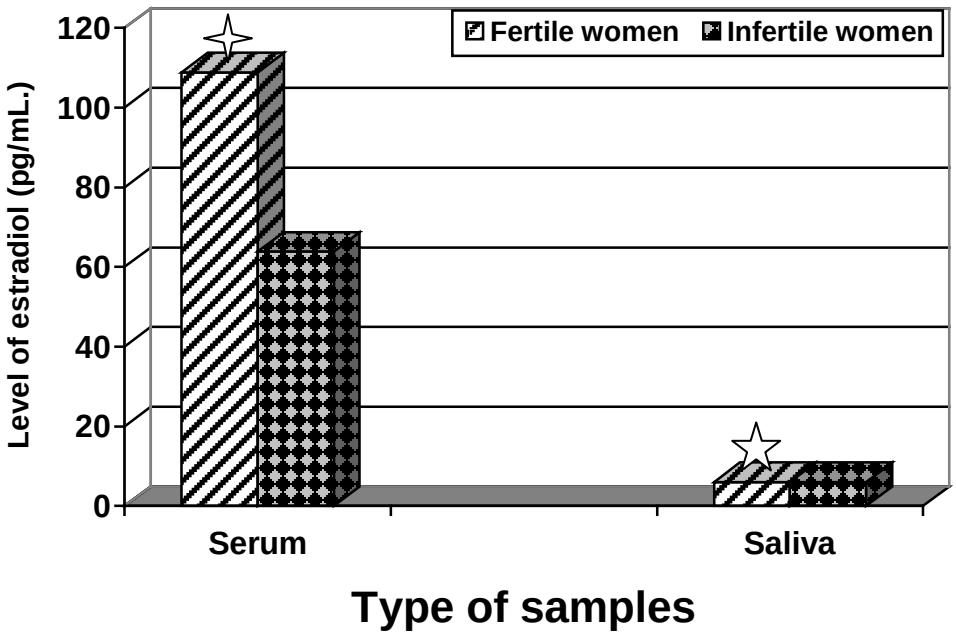

Figure 3-12: Mean level of serum and salivary estradiol for fertile and infertile female

Data are Mean ± SE.

Significant (P<0.05) differences for serum estradiol between fertile and infertile females.

Non significant (P>0.05) differences for salivaary estradiol between fertile and infertile females.

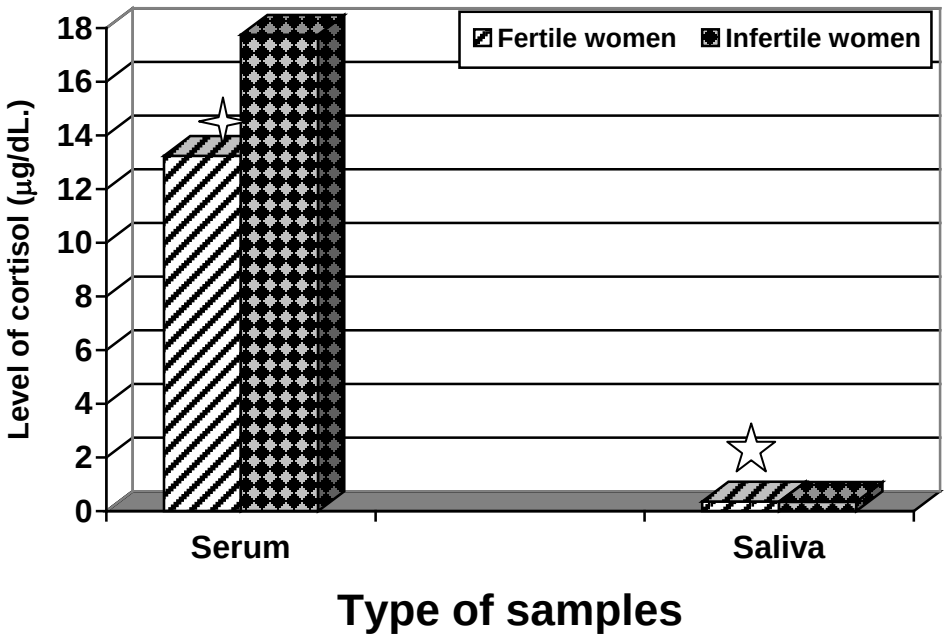

Figure 3-13: Mean level of serum and salivary cortisol for fertile and infertile female*

Data are Mean ± SE.

Significant (P<0.05) differences for serum cortisol between fertile and infertile females.

Non-significant (P>0.05) differences for salivary cortisol between fertile and infertile females.

Table 3-7: Levels of serum and saliva steroidal hormones for females classified according to marital and fertility status

Levels of Hormones	Married (fertile) (No. =16)		Married (infertile) (No. =54)		Single (infertile) (No. =20)	
	serum	Saliva	serum	Saliva	serum	Saliva
Progesterone (ng/mL.)	4.293 ✧✧ ±0.988	0.077 ±0.009	2.356 ✧✧ ± 1.291	0.056 ± 0.004	18.735 ✧ ± 2.206	0.081 ± 0.013
Estradiol (pg/mL.)	74.012 ✧✧ ± 7.394	6.01 ±0.0001	108.98 ✧✧ ± 21.04	6.01 ±0.0001	12.40 ✧✧ ±0.632	6.01 ±0.0001
Cortisol (μg/dL.)	18.735 ✧✧ ± 2.206	0.357 ± 0.003	59.17 ✧✧ ± 5.817	0.360 ± 0.000	16.47 ✧✧ ± 1.183	0.353 ±0.006

- Data are Mean ± SE.
- ✧ Significant (P<0.05) differences between serum and salivary progesterone for single females.
- ✧✧ Highly significant (P<0.01) differences between serum and salivary progesterone for married females.
- ✧✧ Highly significant (P<0.01) differences between serum and salivary estradiol for single females.
- ✧✧ Highly significant (P<0.01) differences between serum and salivary estradiol for married females.
- ✧✧ Highly significant (P<0.01) differences between serum and salivary cortisol for single females.
- ✧✧ Highly significant (P<0.01) differences between serum and salivary cortisol for married females.

Significant ($P<0.05$) elevation was assessed in the level of serum progesterone for single infertile females as compared to other females groups. However, least level of serum progesterone was registered for married infertile females (Figure 3-14). From same figure, little differences were reported in the level of salivary progesterone for three groups of females.

Same results were obtained for salivary E_2 for all groups of females. Conversely, married infertile females have the highest level of serum E_2 as compared to other groups, while the least levels of serum E_2 were observed for single infertile females (Figure 3-15). Similar results were assessed for level of serum and salivary cortisol (Figure 3-16).

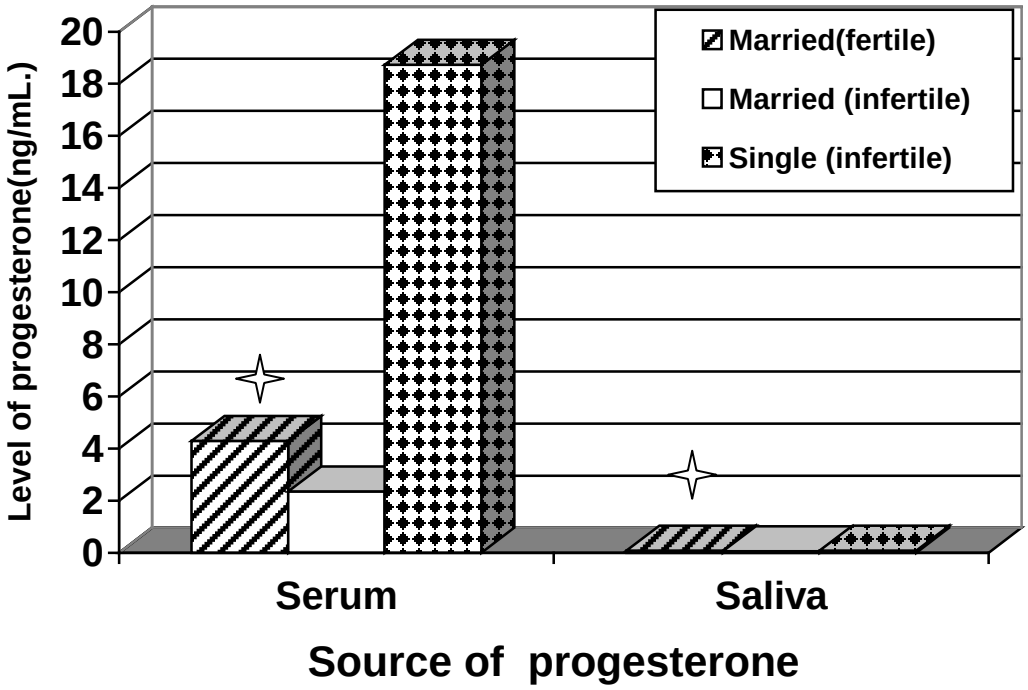

Figure 3-١٤: Mean levels of serum and salivary progesterone for females classified according marital and fertility status

Data are Mean ± SE.
Significant (P<0.05) differences for serum and salivary progesterone for three groups of females.

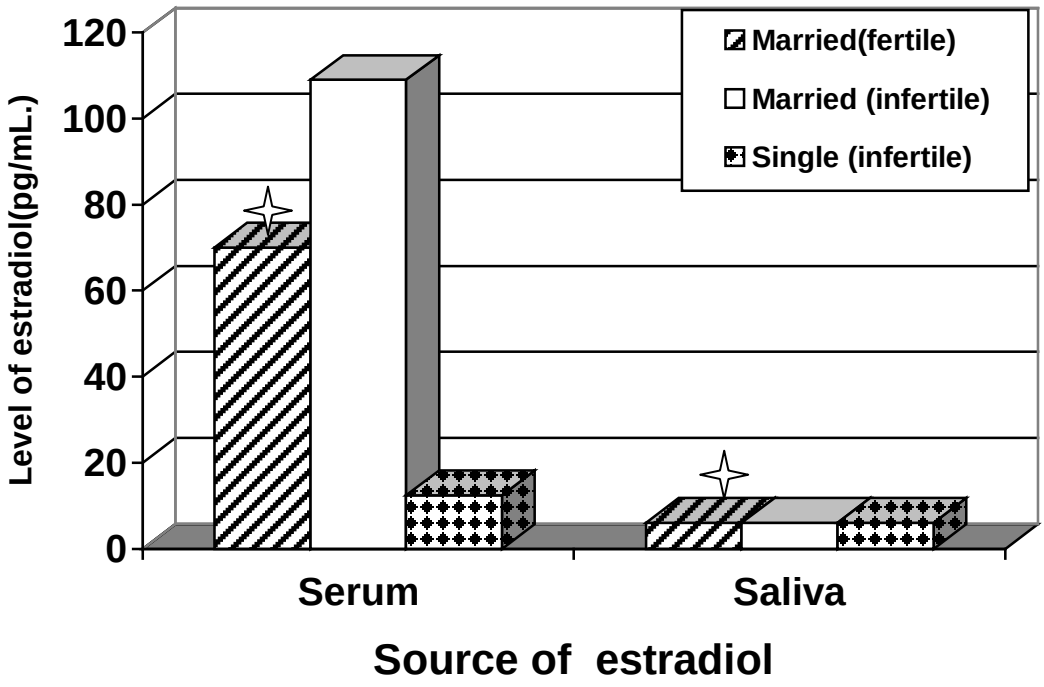

Figure 3-١٥: Mean levels of serum and salivary estradiol for females classified according marital and fertility status

Data are Mean ± SE.

Significant (P<0.05) differences for serum and salivary estradiol between three groups of females.

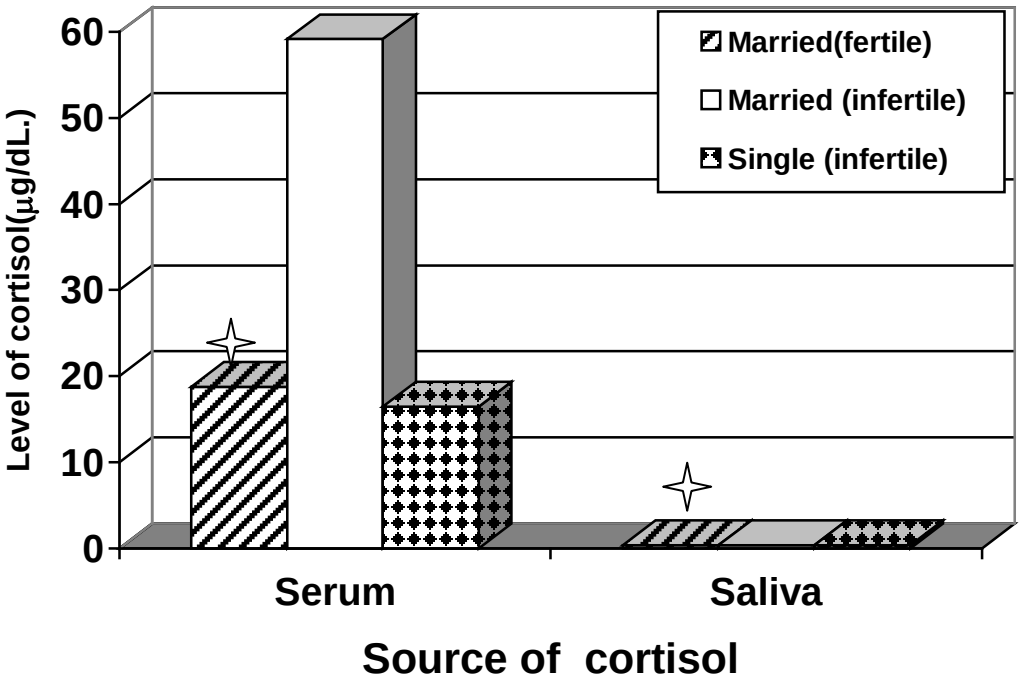

Figure 3-١٦: Mean levels of serum and salivary cortisol for females classified according marital and fertility status

Data are Mean ± SE.
✧ Significant (P<0.05) differences for serum and salivary cortisol between three groups of females.

Review of literatures

1.1 Physiology of hormones

The endocrine system is a control system of organs that secrete hormones which circulate within the body via the bloodstream to affect other cells within specific organs (Hadley, 2000). These hormones that are chemical messenger from one cell (or group of cells) to another that are called target tissue (Mathews and Van Holde, 1990), have a key role in regulating the functions of the body, including reproduction, metabolism, growth and development, water and electrolyte balance, and behavior (Guyton and Hall, 2001).

1.1.1 Non-steroidal Hormones

Most of the hormones in the body are polypeptides and proteins that range in size from small peptide consist of three amino acids to proteins of 200 amino acids (Norman and Litwack, 1987). The proteins and peptides are synthesized from amino acids according to messenger ribonucleic acid (mRNA) template (Dobson, 2000), which is itself synthesized from a deoxyribonucleic acid (DNA) template inside the cell's nucleus in the same fashion as most other proteins synthesis on rough endoplasmic reticulum of endocrine cells (Lodish *et al*., 2004).

Peptide hormone precursors preprohormones (which are biologically inactive) are first synthesized then processed in several stages in the endoplasmic reticulum (Baulieu, 1991), including removal of the N-terminal signal sequence and sometimes glycosylation, resulting in prohormones (Griffin and Ojeda, 2000). The prohormones transferred to Golgi apparatus to package into membrane-bound secretory vesicles, which can be secreted from the cell by exocytosis in response to specific stimuli (Beato *et al*., 1996).

1.1.2 Steroidal Hormones

These hormones are synthesized from cholesterol in the gonads and adrenal glands (Hammes, 2003) as shown in figure (1-1). These are highly lipid soluble molecules that they are simply diffuse across the cell membrane and enter the interstitial fluid and then to the blood (Feder, 1981). Steroidal hormones have an important role on reproductive system representing in hypothalamic-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) is secreted in an episodic fashion from the hypothalamus to activate the production of gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) from the anterior pituitary (Santoro *et al*., 1988).

LH and FSH are released in a pulsatile manner to act at the gonad to control both gametogenesis (spermatogenesis or oogenesis), as well as steroidogenesis (Santoro *et al*., 1986). The major sex hormones estradiol, progesterone, and testosterone are secreted in response to gonadotropins and, in turn, feedback at the level of the hypothalamus and pituitary to control normal reproductive function (Segars and Driggers, 2002).

1.2 Female steroidal reproductive hormones

In human females, there are several steroidal hormones that are involved in reproduction. These hormones are progesterone (Figure 1-2) that is derived directly from pregnenolone, estradiol (Figure 1-3), estroil, esterone that are derived form estrogens. Cortisol (Figure 1-4) is a dominant glucocorticoid synthesized from progesterone, and testosterone which is an androgen hormone produced from progesterone (Kosfeld *et al.*, 2005).

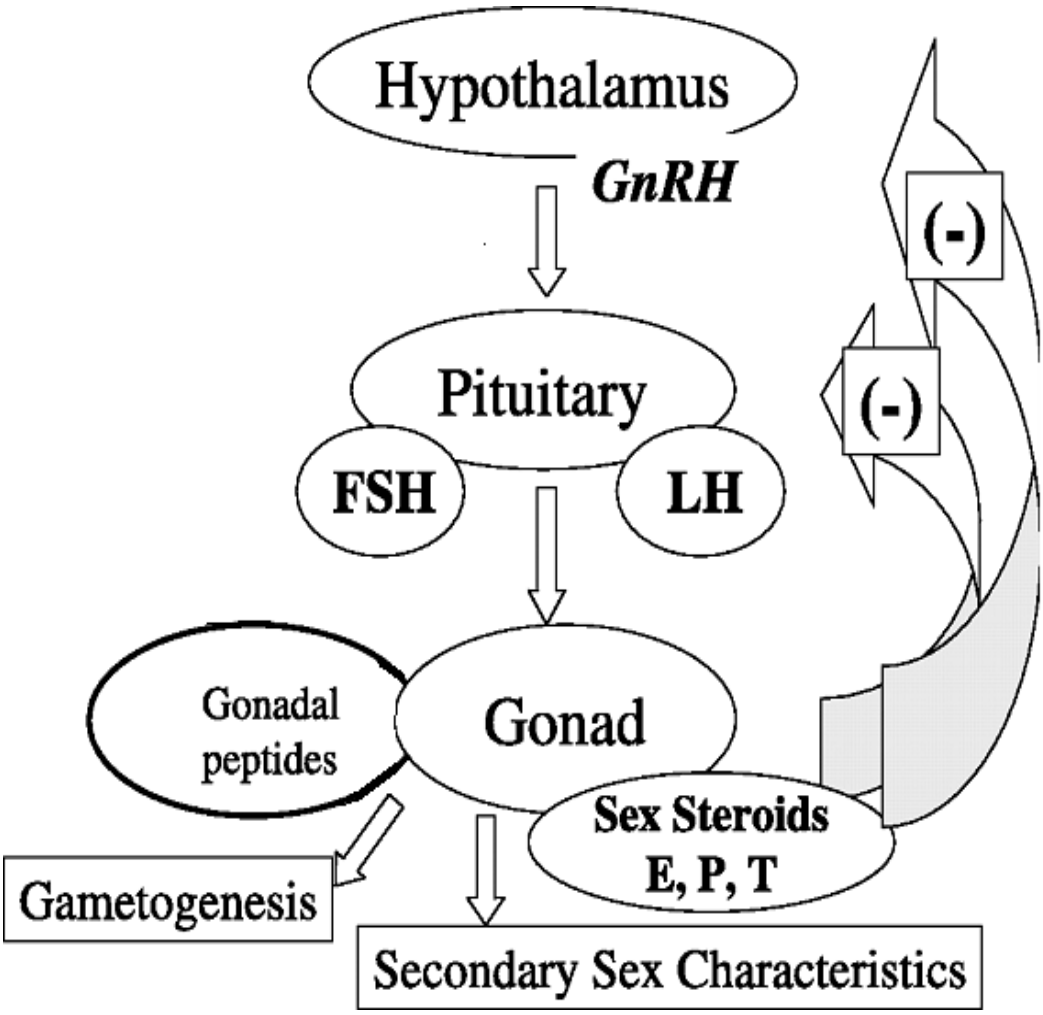

Figure (1-1): The hypothalamic-pituitary-gonadal axis. GnRH,gonadotropin -releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E, estrogen; P, progesterone; T, testosterone (Santoro *et al*., 1988).

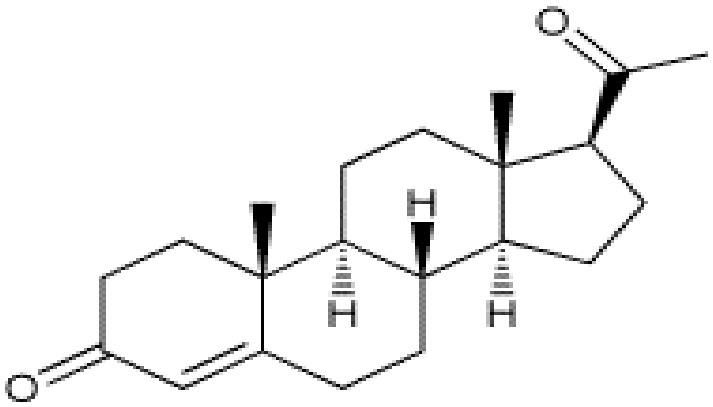

Figure (1-2): Chemical structure of progesterone (Raber, 1999).

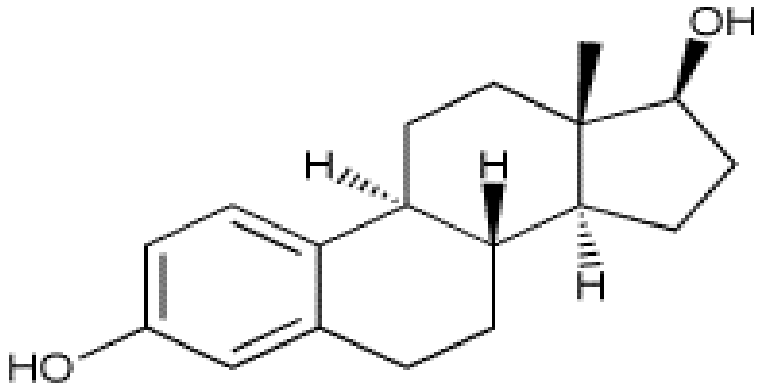

Figure (1-3): Chemical structure of estradiol (Raman and Schlegel, 2002).

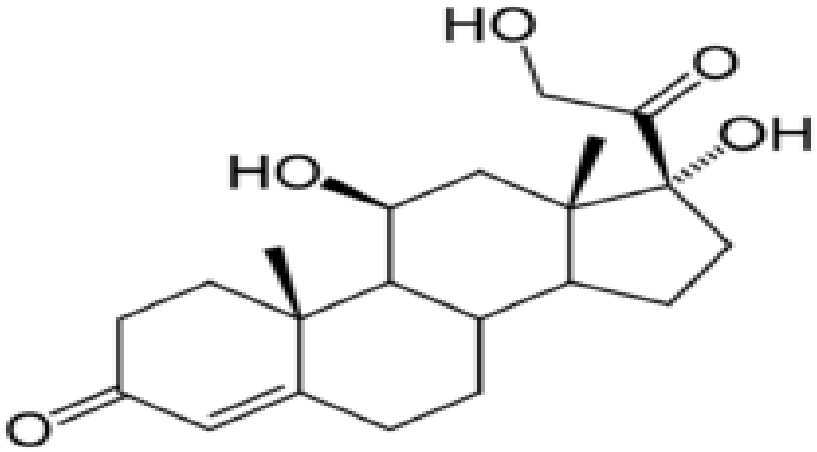

Figure (1-4):Chemical structure of cortisol (Schimmer and Parker, 2007).

1.2.1 Progesterone

The hormone of maturation, associated with estrogen in preparing the endometrium for implantation of the fertilized ovum and the mammary glands for milk secretion (Lydon *et al*., 1995). Progesterone like estrogen is steroids and they are both synthesized in the ovaries from cholesterol or acetyl coenzyme A (Juan *et al*., 2004). In non- pregnant women, progesterone is secreted mainly by the corpus luteum during the latter half of each ovarian cycle while the progesterone in pregnant secreted from placenta that becomes the major source of this hormone and the minor source are adrenal cortex in both sexes and also testes in males (Norman and Litwack, 1987).

In women, progesterone levels varies from 2 to 10 ng/mL after ovulation during the luteal phase, whereas progesterone levels during the pregnancy start to rise and may reach 100-200 ng/mL (Schumacher *et al.*, 2004). Progesterone inactivate estrogen by increasing the sulfurylation of estrogen that is called estrogen sulfotransferase, the attenuation of estrogen action in endometrium done in at least three ways: (1) by reducing the rate of synthesis of estrogen receptors, (2) by bringing about reduction in the intracellular level of estradiol (through conversion to estrone).

Like other steroids, progesterone consists of four interconnected cyclic hydrocarbons (Willard, 2005). Progesterone contains ketone and oxygenated functional groups, as well as two methyl branches as shown in figure (2). Like all steroid hormones, it is hydrophobic; this is mostly due to its lack of very polar functional group (Baron and Hylemon, 1997).

1.2.2 Estradiol

It a sex female hormone but it's also present in males. It represents the more potent estrogens type that contain in addition to estradiol, estrone, and estriol (Pentikainen *et al.*, 2006). During the reproductive years, most estradiol in women is produced by the granulosa cells of the ovarian follicle by the aromatization of androstenedione (produced in the theca folliculi cells) to estrone, followed by conversion of estrone to estradiol (Sharpe and Skakkebaek, 1993). Also produced by conversion of precursor hormones specifically testosterone (Carani *et al*., 1997). Smaller amounts of estradiol are also produced by the adrenal cortex and in men by the testes, in addition to that esrtadiol also produced in brain and in arterial walls (Raman and Schlegel, 2002).

Several studies showed that in the normal menstrual cycle estradiol levels measure typically <50 ng/mL and rise with follicular development, however, estradiol level drop briefly at ovulation, and rise again during the luteal phase for a second peak (Hulshoff *et al*., 2006). At the end of the luteal phase, estradiol levels drop to their menstrual levels this happened in the absence of pregnancy (Woodruff and Mather, 1995). During pregnancy, estrogen levels including estradiol rise steadily, the source of these estrogens is the placenta that aromatizes hormones produced in the fetal adrenal gland (Collins *et al*., 1995). Marshall *et al*. (1991) mentioned that estradiol have several physiological actions includes: (1) stimulate secondary sex characters of females; (2) prepare uterus for spermatozoa transport, (3) increase vascular permeability and tissue edema; (4) stimulate growth and activity of mammary glands and endometrium.

1.2.3 Cortisol

Is a corticosteroid hormone produced by the adrenal cortex in the adrenal gland, which consists of three layers: zona glomerulosa, zona fasiculata, and zona reticularis (de Weerth *et al*., 2003). The synthesis of cortisol takes place in the zona fasciculata that secretes glucocorticoids, cortisol and corticosterone, in addition to small amount of adrenal androgens and estrogens (Scott, 2002). The secretion of adrenal cortex cells is controlled by the hypothalamic-pituitary axis via adrenocorticotropic hormones (ACTH). Production of ACTH is in turn stimulated by corticotropin-releasing hormone (CRH), released by the hypothalamus, the ACTH cause secretion of cortisol from adrenal (Palacios and Sugawara, 1982).

Weber (1998) reported that cortisol causes stimulation in glucogneugensis 6-10 fold by increasing the enzymes required to convert amino acids into glucose in the liver cells, and causes mobilization of amino acids from the extrahepatic tissues from muscle this will increase amino acids concentration in the plasma to enter glucogneugensis process of the liver and promote the formation of glucose. Cortisol also stimulates mobilization of fatty acids from adipose tissue, this increase concentration of fatty acids in the plasma and increases their utilization for energy (Guyton and Hall, 2001). The hormone cortisol called the "stress hormone" as it is involved in the response to stress and is used to treat allergies and inflammation (de Weerth *et al*., 2003).

1.3 Actions of steroidal reproductive hormones

1.3.1 Progesterone

Receptors for progesterone are expressed as two distinct isoforms, PR-A and PR-B that arise from a single gene (Kastner *et al.*, 1990; Conneely *et al.*, 2003). However, the ratios of the individual isoforms vary in reproductive tissues as a consequence of developmental (Shyamala *et al.*, 1998) and hormonal status (Duffy *et al.*, 1997) and during carcinogenesis (Brandon *et al.*, 1993; Graham *et al.*, 1996).

Progesterone receptor has a modular protein structure consisting of distinct, functional domains capable of binding steroidal ligand, dimerizing liganded receptors, interacting with hormone-responsive DNA elements, and interacting with co-regulator proteins required for bridging receptors to the transcriptional apparatus (Tsai and O'Malley, 1994; Giangrande and McDonnell, 1999).

Binding of progestin agonists induces conformational changes in receptor structure that promote interaction of coactivator proteins with distinct activation function domains (AFs) located within both the amino- and carboxy-terminal regions of the receptor (McKenna *et al.*, 1999). Wen *et al.* (1994) suggested that such coactivators promote chromatin remodeling and bridging with general transcription factors, resulting in the formation of productive transcription initiation complexes at the receptor-responsive promoter. In contrast, binding of receptor antagonist compounds induces receptor conformational changes that render AFs non permissive to coactivator binding and instead promote interaction with co-repressor proteins that inhibit the receptor's transcriptional activity (Tsai and O'Malley, 1994).

The ability of progesterone to interact with a variety of coactivator and co-repressor proteins, together with the differing expression of co-regulators,

explain a key role of these proteins in mediating different tissue-specific responses of progesterone receptors to steroidal ligand (Rowan *et al.*, 2000). Progesterone receptors also can be activated in the absence of steroidal ligand by phosphorylation pathways that modulate their interactions with co-regulator proteins (McKenna *et al.*, 1999).

Difference between PR-A and PR-B isoforms in that the PR-B protein contains an additional sequence of amino acids at its amino terminus, and this PR-B-specific domain encodes a third transactivation function (AF3) that is absent from PR-A (Sartorius *et al.*, 1994). Recent evidence studies has demonstrated that AF3 allows binding of a subset of coactivators to PR-B that is not efficiently recruited by progestin-bound PR-A (Giangrande *et al.*, 2000). The PR-A isoform is necessary to oppose estrogen-induced proliferation as well as PR-B-dependent proliferation (Feigelson *et al.*, 2004).

When no binding hormone is present the carboxyl terminal inhibits transcription (Dossus *et al.*, 2006). Binding to a hormone induces a structural change that removes the inhibitory action. However, after progesterone binds to the receptor, restructuring with dimerization follows and the complex enters the nucleus and binds to DNA (Gadkar-Sable *et al.*, 2005). There transcription takes place, resulting in formation of messenger RNA that activates cytoplasmatic ribosomes to produce specific proteins (Kase *et al.*, 1999).

1.3.2 Estradiol

Estradiol enters cells freely and interacts with a cytoplasmic target cell receptor (Carani *et al*., 1997). Behl *et al*. (1995) suggested when the estradiol receptor has bound its ligand it can enter the nucleus of the target cell, and regulate gene transcription which leads to formation of messenger RNA. The mRNA interacts with ribosomes to produce specific proteins that express the effect of estradiol upon the target cell (Collins *et al*., 1995).

There are two different forms of the estrogen receptor, usually referred to as α and β, each encoded by a separate gene ESR1 and ESR2 respectively (Pentikainen *et al*., 2006). Hormone activated estrogen receptors form dimers, and since the two forms are coexpressed in many cell types, the receptors may form ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers (Raman and Schlegel, 2002). Estrogen receptor alpha and beta showed significant overall sequence homology, and both are composed of seven domains, listed from the N- to C-terminus (Li *et al*., 2004).

Estradiol binds well to both estrogen receptors, ERα and ERβ, in contrast to certain other estrogens, notably medications that preferentially act on one of these receptors; these medications are called selective estrogen receptor modulators, or SERMs (Andrew, 2000).

1.3.3 Cortisol

Cortisol binds with high affinity to glucocorticoid receptors (GR) which is a ligand-activated transcription factor (Lu *et al*., 2006). Pratt *et al*. (2006) reported that, in the absence of hormone, GR resides in the cytosol complexed with a variety of proteins including heat shock protein 90 (hsp90), heat shock protein 70 (hsp70) and the protein FKBP52 (FK506-binding protein 52). The endogenous glucocortiod hormone cortisol diffuses through

the cell membrane into the cytoplasm and binds to the GR resulting in release of the heat shock proteins (Buckingham, 2006).

The active form of GR has a direct mechanism of action in which homo dimerization of the receptor, translocation via active transport into the nucleus, and binding to specific DNA responsive elements activating gene transcription, the biologic response depends on the cell type. However this mechanism of action is referred to as transactivation (Hayashi *et al*., 2004). In the absence of activated GR, other transcription factors such as nuclear factor-kappa B (NF-κB) or activator protein 1 (AP-1) themselves are able to transactivate target genes (Mendonca *et al*., 2002). However activated GR can complex with these other transcription factors and prevent them from binding their target genes and hence repress the expression of gene normally upregulated by NF-κB or AP-1. This indirect mechanism of action is referred to as transupression (Neeck *et al*., 2002).

1.4 Serum

Serum is clear, yellowish liquid component of blood that remains after clotting has occurred and fibrous, insoluble material has been removed (Blankenship *et al*., 2003). Before clotting occurs, the liquid of the blood is called plasma, both plasma and serum contain proteins, salts, sugars, waste products, vitamins, minerals, fats, and hormones (Adkins *et al*., 2002). But plasma also contains the protein fibrinogen and certain other elements necessary for clotting, and it therefore clots as easily as whole blood (Sonel *et al*., 2000). Serum lacks these elements, so it does not clot.

Serum from animals like horses and sheep can be used to provide human beings with protection against infections or poisons (Keller and Stiehm, 2000). Animals are given specific protein molecules derived from living organisms like bacteria to stimulate animal immune systems to produce

antibodies, once injected into a human patient, a serum containing animal antibodies can provide rapid immunity against specific diseases, such a serum is called an antitoxin, and is used to treat such diseases as diphtheria (Mupapa *et al.*, 1999).

1.5 Saliva

Saliva is a dilute aqueous fluid (osmolality less than or equal to that of plasma) originating from the salivary glands (Figure 1-5) located under the tongue and along the sides of the mouth (Douglas, 2000).

It is a complex mixture of mucins, enzymes, antibodies, electrolytes, and hormones. It serves various functions including digestion, lubrication and protection of the oral mucosa (Depaolo *et al.*, 1999). Risk for dental caries increases as saliva production decreases, as seen during sleep, as a symptom of disease, or as a side effect of fasting or medication (Johansson *et al.*, 1992).

Bad nutrition in the early stage of development and chronic effects of malnutrition can impair the salivary gland's secretary function, also salivary function may be damaged by radiotherapy for oral cancer (Rugg-Gunn *et al.*, 1981).

1.5.1 Composition of saliva

Water consist 98% of saliva component, but it also contains many important substances, including electrolyte such as calcium (0.88-2.05 mmol/l), chloride (5-40 mmol/l), magnesium (0.08-0.56 mmol/l), potassium (6.4-37 mmol/l), various enzymes such as α-amylase (11900-305000 U/l), lysozyme (6-12 U/l), antibacterial compounds such as IgA (42- 174 mg/L) (Birkhed and Heintze, 1989).

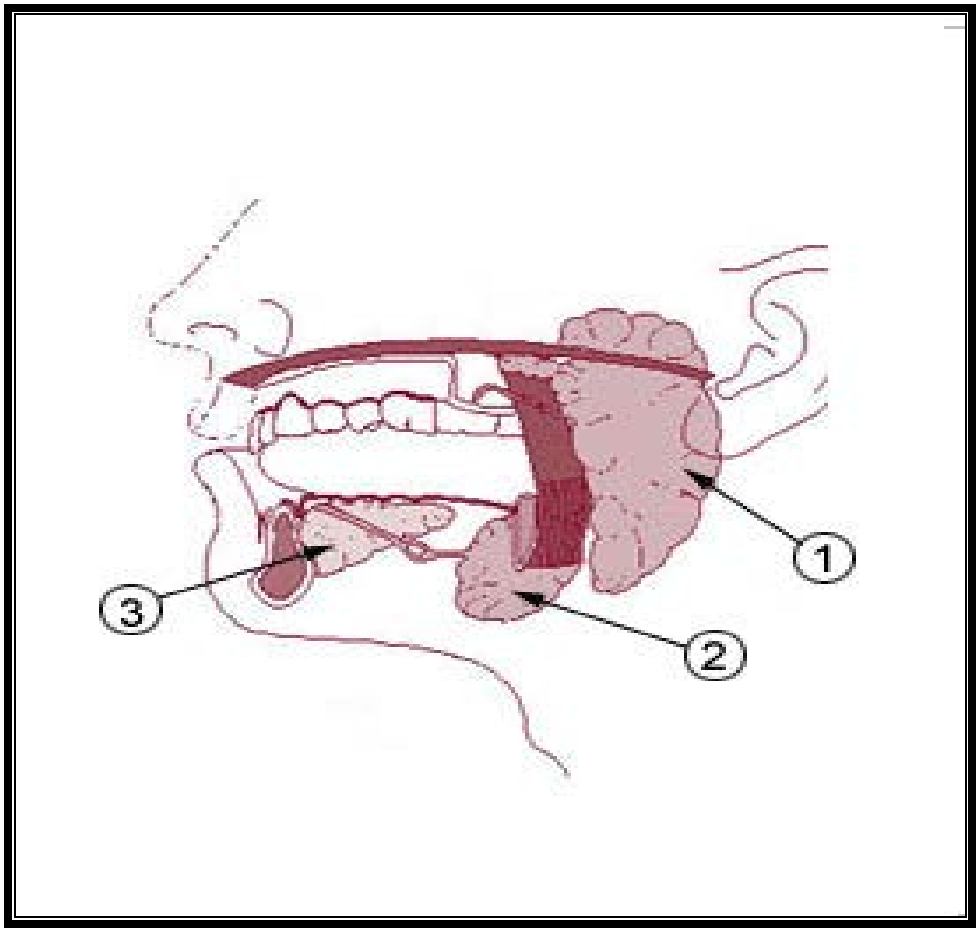

Figure (1-5): Salivary glands: (1) Parotid gland, (2): submandibular gland, (3): Sublingual gland (Wong and Georgy, 2006).

The electrolyte consists of principle ions such as sodium, potassium, chloride and bicarbonate (Jacobson, 1981). It was noted that saliva supplies enzymes for digestion such as α-amylase, which digest dietary starch into maltose (Thompson *et al*., 1987). Luiz and jose (2003) indicated that the presences of antibacterial compounds especially the immunoglobulin A make saliva play an important role in protect mouth from bacteria.

Saliva also contain free steroid hormones that is not bound to steroid binding protein, these hormones consist 1-5% of steroids which in blood are bound to proteins such as sex hormone binding globulin (SHBG), cortisol binding globulin (CBG), and albumin (Read, 1993). Mucus is abundant and important component of saliva, giving it virtually lubricating properties. Mucus also gives saliva viscosity which consists of mucopolysaccharides and glycoprotein in saliva (Vining and McGinley, 1985; Iontcheva *et al*., 1997).

1.5.2 Presence and movement of steroidal hormones between serum and saliva

In blood, 92-95% of the steroids are bound up by binding proteins such as SHBG, CBG, and albumin (Vittek *et al*., 1985). Only small fraction of steroids not bound is considered the free fraction or being bioavailable in blood, however these molecules can enter the capillary beds of tissues (Read, 1993). The free or bioavailable steroids enter the salivary glands and salivary duct by passive diffusion in the same way of entering to other tissues of the body (Collins *et al*., 1995).

The transfer of steroids from serum to saliva depends on small molecular weight of these molecules (less than 400 Daltons), and great lipid solubility that enables them to freely diffuse from blood to saliva (Lipson and Ellison, 1989). Saliva concentration of a particular hormone is dependent on the affinity and total binding capacity of various binding proteins in plasma (Malamud, 1992). Mandel (1990) concluded that as blood passes through

salivary glands, free "unbound" and weakly bound (low affinity binding protein) forms of hormones will diffuse through the salivary gland epithelium into the saliva.

As in other clearing organs, membrane transfer occurs in both directions, is passive for most substances and equilibrium is governed by the trans-membrane concentration gradient (Frisch, 1987). Thus, saliva levels reflect the free concentration of hormones in plasma, and in the absence of high affinity, high capacity-binding proteins, these levels correlate with plasma concentrations (Wong *et al*., 1990). On the other hand, hormones that have high affinity, high capacity-binding proteins, such as thyroxine, are difficult to assay in the saliva because these hormones have very high plasma total to free hormone ratio and exist in small amounts in saliva (Ferguson, 1984).

1.6 Female reproduction

1.6.1 Oogenesis

During early embryonic development, primordial germ cells migrate from the dorsal endoderm of the yolk sac along the hindgut to the genital ridge and then undergo mitotic proliferation (Moore and Persaud, 1998). Following which oocytes begins meiosis and arrest in prophase of first meiotic division (M1) during the forth to the seventh month of fetal development till puberty (Byrd, 1998).

Bendsen *et al.* (2006) reported that at the fifth month of intra-uterine life the total number of the germ cells reaches its maximum, estimated at 7 millions. The total cortical content of germ cells falls to 1-2 millions by birth as a result of prenatal oocyte depletion (Himelstein-brew *et al.*, 1976). At the onset of puberty, the germ cell mass has been reduced to 300 000 units and only 400-500 (less than 1%) will be ovulated in the reproductive lifetime of the individual (Guyton and Hall, 2001).

1.6.2 The ovaries

An ovary is an egg-producing reproductive organ found in the mammalian females. It is often found in pairs as part of the vertebrate female reproductive system (Johnson *et al.,* 2004). In the ovary, the reproductive unit is a follicle, which consists of a germ cell surrounded by endocrine cells. Active follicular growth and atresia in the ovaries occur already during infancy and childhood (Fauser and van Heusden, 1997). In response to rising levels of gonadotropins, especially FSH, ovarian follicles undergo final maturation at puberty (Fauser and van Heusden, 1997, Campbell and Monga, 2000).

Ovarian follicles are the basic units of female reproductive biology; they are roughly spherical aggregations of cells found in the ovary. They contain a single oocyte called ovum or egg (Baker, 1982). Oocytes are surrounded by layer of granulosa cell that are together enclosed in a thin layer of extracellular matrix, the follicular basement membrane or basal lamina to constitute the ovarian follicle (McGee and Hsueh, 2000).

1.6.3 Development stage of ovarian follicles: -

1.6.3.1 Primordial follicle

In the postnatal ovary, the primordial follicles contain immature oocytes that are large, spherical about 35µm in diameter surrounded by flat, squamous granulosa cells that are segregated from the oocyte's environment by the basal lamina (Fortune *et al.,* 2000). The oocyte and its cellular investment constitute the primordial follicle, each of which is about 30-60 µm in diameter (Yen *et al.*, 1999).

1.6.3.2 Growing follicle

When the primordial follicles enter the growth phase, they enlarge by an increase in the size of the oocyte together with granulosa cell proliferation so called primary follicle (Johnson *et al.,* 2005). Following that, the enteric layers designated the theca interna, (closest to the basal lamina) and theca extena (the

outer portion). The theca layers appear when granulosa cells proliferation produce 3-6 layers of cells (Ganong, 2003)**.**

Formation of the tertiary follicle is associated with further hypertrophy of the theca cells and the development of the antral cavity (at a follicular size 100-200 μm) which divides granulosa cells into cells surrounding the oocyte (cumulus oophorus) and cells that border the basement membrane (Bart and Anne, 1997). The fluid in the antral cavity so called follicular fluid consists of plasma filtrate and secondary products of granulosa cells, some of which are found in concentrations greater than the peripheral blood (Yen and Adashi, 1999).

Immature follicles grow in the ovaries through FSH stimulation, while LH causes final maturation of oocytes and triggers ovulation (Ganong, 2003). During growth of the follicles, mainly estrogen is secreted (Guyton and Hall, 2001). After ovulation, the secretory cells of ovarian follicles and theca cells develop into a structure called corpus luteum under effect of LH hormone (Moore and Persaud, 1998), this structure secret large quantity of both female hormones, progesterone and estrogen (Baird, 1991; Carr, 1998). However, level of ovarian hormones estrogen and progesterone decline due to degeneration of corpus luteum and menstruation begin (Campbell and Monga, 2000).

1.6.3.3 Mature Graafian follicle

During its growing phase, the volume of the oocyte will increase to become one of the largest cells in the body (Gougeon, 1996). As a result of the accumulation of liquids, the follicular cavity increases in size and the oocyte adheres to the wall of the follicle through the pedicle (cumulus oophorus) formed by the granulosa cells (Luiz *et at.*, 1998).

Graafian follicle is about 20 mm in diameter and can be seen as a vesicle that bulges from the surface of the ovary and at this stage of development. The

mature Graafian follicle is ready to release the ovum during ovulation (Carr, 1998). Resumption of metabolic activity depends upon subsequent fertilization, if the latter does not occur within 6-24 hours following ovulation, the oocyte will degenerate (Edwards, 1980).

1.7 Human menstrual cycle

It is spontaneous, cyclic, predictable and regular vaginal bleeding called the female monthly sexual cycle (Carr, 1998). The hypothalamus, pituitary, ovaries and genital tract are all involved in the regulation of normal menstrual cycles, which initially begin during gonadarche at puberty. The mean or median age at the onset of menstruation varies within 12.0-15.0 years (Mishell, 2001). The median length of the ovulatory period in healthy fertile women is 28 days with a range of 25 to 32 days (Polson *et al.*, 1988).

1.7.1 Phases of menstrual cycle

1.7.1.1 Follicular phase

Early in the menstrual cycle, a varying number of follicles, typically 5-8 will be recruited under the influence of a rise in follicle stimulating hormone (FSH) (Speroff and Fritz, 2005). These follicles that have been growing in a process known as folliculogenesis compete with each other for dominance (David, 2004). The selection of a single follicle destined to ovulate is associated with a high capacity for androgen, estrogen, progesterone and inhibin B biosynthesis and secretion (Campbell and monga, 2000).

The maturation of dominant follicle takes place at cycle day 8-12 and ultimately ovulation at cycle day 13-15 (Yen and Adashi, 1999). All but one of these follicles will undergo atresia, while one (or occasionally two) dominant follicles will continue to maturity. As they mature, the follicles secrete increasing amounts of estradiol, and estrogen (Nadal *et al.*, 2001). That means rising in estrogen level and parallel the growth of follicle and the

number of granulosa cells, estrogen together with FSH induce the formation of LH receptors on granulosa cells **(**Ganong, 2003**).**

1.7.1.2 Ovulation

The release of mature oocytes from the ovary requires weakens and degradation of the follicle wall, as well as the overlying ovarian tissues. This happens through the expression of a series of critical genes, triggered by the rise of preovulatory LH level (Tsafiri *et al*., 1993). This rising occurs under the control of estradiol which is secreted in increasing amount by the dominant follicle (Knobil and HotchKins, 1988).

About 34-36 hours after the onset of LH surge, ovulation occurs with the release of the ovum from the follicle (Hoff *et al.*, 1983). Where it is caught by the fimbriae at the end of the Fallopian tube (also called the oviduct). After entering the oviduct, the ovum is pushed along by cilia, beginning its journey toward the uterus (Baewald *et al.*, 2004). At this time the LH surge induce resumption of meiosis, lutinization of the follicular cells, as well as the final steps of a cascade of intraovarian events (Tsafiri *et al.*, 1993).

The fall in LH following ovulation is thought to be secondary to the loss of a positive feedback due to the decline in estrogen level or depletion of LH content of the pituitary (Carr, 1998). If no fertilization occurs, the oocyte will degenerate approximately twenty-four hours after ovulation (Chabbert-Buffet *et al.*, 1998).

1.7.1.3 Luteal phase

Following ovulation, the follicle undergoes changes in structure and function. When the process of lutinization is completed, the corpus luteum is established (Diaz *et al*., 1992). The corpus luteum secretes progesterone and prepares the estrogen primed endometrium of a fertilized ovum and maintains early pregnancy (Ganong, 2003). In case fertilization and implantation of the embryo do not occur, the corpus luteum regresses after 14 days, and

eventually replaced by fibrous tissue to form the corpus albicans (Cabbert-Buffet and Bouchard, 2002).

As the corpus luteum dies the estrogen and progesterone levels fall precipitously, endometrium regresses, ischemia and tissue damage occur, shedding of the functional endometrium (stratum compactum and stratum spongiosum) takes place (Campbell and Monga, 2000). Within same time FSH level rises allowing the initiation of a new cycle (Le *et al.*, 1993). In addition to that, the uterus, the cervix, vagina, breast and even the psyche of the women all have cyclic changes in response to these hormonal fluctuations (Ganong, 2003).

1.8 The use of immunoradiometric assay for assessment of salivary hormones

Immunoradiometric (IRMA) technique is a scientific and precise method used to test hormonal levels in the blood involving the use of radioactive materials which specifically binds to biological substances (Chakrabarti *et al.*, 1985). IRMA techniques for the determination of compounds with a low molecular weight, like steroid hormones, have been available for the past decade (Hoffmann, 1981).

Today, millions of assays are performed every year in research and clinical chemistry laboratories (Dixon and Heitzman, 1981). It should be noted that many important and sometimes life saving therapeutic measures are based on data derived from IRMA and, recently using a similar assay technique enzyme-immunoassay (Hoffmann and Oettel, 1976).

In general, measurements are performed on body fluids, predominantly blood plasma or serum. Presently, there is no other assay technique that can compete with RIA relative to reliability (i.e., specificity, precision, accuracy, reproducability), sensitivity and assay speed (Hoffmann, 1983).

It is a technique extensively used in all endocrinological investigations, utilizes the double antibody procedure for the separation of bound hormone. This method of separation is time consuming and requires additional reagents. Solid phase RIA's are developed in many laboratories, using antisera (a/s) immobilised on sephadex, cellulose (Leif Wide, 1981; Chakrabarti *et al.*, 1985). Attempts have been made to improve this system using antibody adsorbed on plastic tube, further eliminating a centrifugation step (Murthy and Moudgal, 1986).

This technique used to assist salivary hormones which considered being a highly reliable technique (Dabbs, 1991). The results of salivary testing are also probably more meaningful than blood testing, because they measure the level of active, free hormone, while blood testing, on the other hand, measures also hormones that is bound to plasma proteins, thus measuring bound/inactive hormones as well (Riad-Fahmy et al., 1982).

Subjects, Materials and methods

2.1 Subjects

Ninety fertile and infertile females were enrolled in this study. All these females were attending the Institute of Embryo Research and Infertility Treatment, Al-Nahrian University and Al-Kadhmiya Teaching Hospital-Ministry of Health.

Samples were collected from females during the period from December 2006 to July 2007; with age range from 16 – 55 years, and the mean of age was (35.5±1.8). The informations about each subject were taken from patients using information sheet which is prepared as shown in table (1).

Table (2-1): Information sheet for each subject

Female status: **Healthy** **Infertile**

General information	**Sample no.:**
Name:	File no:
Type of infertility:	Child no:
Duration of infertility:	Birth:
Cycle day:	Occupation:
Cycle length:	Blood group:
Notes:	Weight:

Hormone levels	**Serum**	**Saliva**	Notes
Progesterone (ng/mL.)			
Estradiol (Pg/mL.)			
Cortisol (µg/dL.)			
Others			

Female factor infertility:
Marital status:

2.2 Materials

Table (2-2) Tools and equipment used in this study

	Instrument	Company	Origin
1	Centrifuge	Hitich	Germany
2	Gamma counter	Wallac	Finland
3	Kit for measuring hormones	Immunotech	French

2.3 Collection of samples

2.3.1 Serum

A venous blood (5mL) was collected in clean disposable plastic tube. Serum was isolated from coagulated blood after 8 minutes centrifugation (2500 r/min). 2 mL of isolated serum transferred into another plastic tube to be stored at ─20°C in the freezer until later use for hormonal assay. All samples then were performed by using immunoradiometric assay (IRMA).

2.3.2 Saliva

Subjects were told not to eat, drink or smoke for 1 hour before sampling. The saliva samples (3 mL) were collected in clean glass tubes, to avoid the interaction of hormones with plastic materials. The saliva was collected from females in restful and quiet circumstances at 8:00-9:00 a.m**,** before brushing, flossing teeth, eating, drinking or applying makeup.

Also they told to rinse their mouth at least twice with cool water. Then chew the sugarless gum provided for 1-2 minutes, swallowing saliva as usual. It is possible to continue chewing the gum during saliva collection but not spit it into the collection tube. Remove the cap of the

saliva collection tube and spit directly into the tube, avoid touching the mouth of the tube with hands. Fill the tube at least 3/4 spit and caps the tubes, making sure the cap is on evenly and securely. It took them a few minutes to generate sufficient saliva.

Centrifuge to remove debris from saliva that was spitted into test tubes and were kept in −20ºC immediately after collection until to be analyzed with immunoradiometric assay.

The serum and saliva tubes should be remarked with name or number of specimen and date of collection.

2.3.3 Time of sample collection

Blood was drawn and collected from females at any time of day for progesterone and estradiol assays. While for cortisol assay blood must take at 8:00 am or 9:00 am and the females should not be under stress or exhaustion that may affect the results of cortisol analysis.

For unstimulated whole saliva, it was collected for 3-5 minutes by teaching the subject to spit saliva into glass test tube once each minute. Saliva samples were taken between 8:00 am to 9:00 AM and immediately frozen at refrigerator to be later used for hormonal assay.

2.4 Assessment of samples

Levels of hormones were measured in serum and saliva for ninety females using immunoradiometeric assay (IRMA) technique and Wallac apparatus (HVD Life Sciences; LKB Wallac, Turku, Finland) and using immunotech RIA kits (immunotech, a BECKMAN counter company, French).

Prepared standards and control for each hormone, using the Anti-hormones antibody-coated tubes labeled with iodine 125, using the calibrator vials contain concentration of hormones for a standard range from 0 to 60 ng/mL in human serum with sodium azide for progesterone.

For estradiol the standard range from 0 to 2000 pg/mL in human serum with sodium azide, while for cortisol the standard range from 0 to 2000 nM in buffer with bovine serum albumin and sodium azide.

The tracer added to react with hormones, and mixing with vortex-type mixer to ensure the presence of hormones inside the vials, then shaking for about 1 hour horizontally or orbital to compose immune complex between the hormone and anti-hormone, samples then soaking to draw the superabundant of tracer and serum or saliva.

Radioactivities were assessed in Gamma counter for 1 minute, all the analyses were made in duplicate.

2.5 Sensitivity and coefficients of variation

The sensitivity of progesterone, estradiol and cortisol were 0.05 ng/mL, 6 pg/mL and 0.36 µg/mL respectively, Inter and intra-assay coefficients of variation for progesterone was 9.0% and 5.8%; respectively, while for estradiol was 11.2% and 12.1% respectively, and for cortisol was 9.2% and 5.8%; respectively (Immunotech diagnostic kit leaflet, 2006).

2.6 Experimental design

Ninety females were included in this study. From each female, serum and saliva samples were obtained and assessment the levels of steroidal hormones including progesterone, E2 and cortisol. Therefore statistical comparisons were performed for females which divided into groups according to:

1. Age groups.
2. Type of infertility.
3. Marital status.
4. Fertility status.

2.7 Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS; version 11.0). Descriptive analysis was done to obtain mean levels of steroidal hormones from serum and saliva of females that enrolled in this study in which data expressed as Mean ± SEM. analysis of variance (ANOVA) test were applied to compare among different groups of females to assess the mean level of hormones in serum and saliva. Differences between values were consider statistically significant at ($P<0.05$).

Summary

The objective of the present study was to assess the level of steroidal hormones in serum and saliva of females, as well as correlation between serum and salivary steroidal hormones including progesterone, estradiol, and cortisol were assessed. Ninety females were involved in the present study. The age of females was ranged (16-55) with mean age 35.5±1.8 years. These females were attending the Institute of Embryo Research and Infertility Treatment, Al-Nahrian University and Al-Kadhmiya Teaching Hospital-Ministry of Health, during the period from December 2006 to July 2007. Females were classified into four groups according to age, type of infertility, fertility status and marital status. Evaluation of steroidal hormones included progesterone, estradiol, and cortisol in serum and saliva for each female was done using radioimmunoassay technique for all groups of females.

Results of the present study showed that the level of serum and salivary progesterone and cortisol were positively synchronized as elevation and vice versa, while the results for salivary estradiol were not reproducible or sensitive. Results appeared that the classification of females according to age groups there was significant ($P<0.05$) differences in the level of steroidal hormones for serum and saliva among different age groups.

When females were classified according to type of infertility, results showed that there was a significant ($P<0.05$) reduction in the mean levels of salivary steroidal hormones as compared to serum levels for both primary (1^o) and secondary (2^o) infertility.

While the results for females classified according to marital and fertility status, there was a significant ($P<0.05$) differences in the level of steroidal hormones between serum and saliva for single and married females, and also for fertile and infertile females. However, results showed a non significant ($P>0.05$) differences in the level of serum and salivary steroidal hormones between single and married females, and also for serum steroidal hormones between fertile and infertile females. While there was a significant ($P<0.05$) differences in the levels of serum estradiol and cortisol between fertile and infertile females, non significant ($P>0.05$) difference for salivary estradiol and cortisol between fertile and infertile females, there was a non significant ($P>0.05$) differences of serum and salivary progesterone were observed between fertile and infertile females.

Supervisor Declaration

We, certify that this thesis was prepared under our supervision in Al-Nahrain University/College of Science department of Biotechnology as partial requirements for the Degree of Master of Science in Biotechnology.

Assistant Professor Dr.
Muhammad Rafeeq Abdul Majed
Supervisor

Assistant Professor Dr.
Muhammad-Baqir M-R. Fakhrildin
Supervisor

In review of the available recommendation, I forward this thesis debate by the Examination Committee.

Signature:
Prof. Dr. Kadhim M. Ibrahim
Scientific degree: Professor
Head of Biotechnology Department
College of Science
Al-Nahrain University

Committee Certification

We, the committee, certify that we have read this thesis and examined the student in its contents and that according to our opinion is accepted as a thesis for the degree of Master of Science in biotechnology.

Assistant Professor Dr.
Usama Al-Nasiri
(Chairman)

Assistant professor Dr.
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بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

اللهُ يَعْلَمُ مَا تَحْمِلُ كُلُّ أُنْثَى وَمَا تَغِيضُ الأَرْحَامُ وَمَا تَزْدَادُ وَكُلُّ شَيْءٍ عِنْدَهُ بِمِقْدَار*

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(الرعد الآيه ٨)

الخلاصة

تهدف الدراسة ألحاليه إلى تقويم مستوى الهرمونات الستيرويديه (steroidal hormones) في المصل (serum) واللعاب (saliva) للإناث، وكذلك العلاقة في مستوى الهرمونات الستيرويديه التي تشمل البروجيستيرون (progesterone)، والاستراديول (estradiol)، والكورتيزول (cortisol) بين مصل الدم واللعاب.

شملت هذه الدراسة ٩٠ أنثى، كان معدل أعمارهن هو (35.5±1.8 years) تم اختيارهن من الأزواج الذين يعانون من العقم والمترددين إلى معهد أبحاث الأجنة وعلاج العقم في جامعه النهرين وكذلك الإناث المترددات على مستشفى ألكاظميه التعليمي للمدة مابين شهر كانون الأول 2006 إلى تموز 2007.

تم تقسيم الإناث إلى مجاميع اعتمادا على العمر ونوع العقم وحالة الخصوبة والحاله الزوجية. وقد أجري تقييم للهرمونات الستيرويديه الثلاثه (البروجيستيرون progesterone، والاستراديول estradiol ، والكورتيزول cortisol) لكل من المصل واللعاب باستخدام تقنيه الاختبار الإشعاعي المناعي (Radioimmunoassay).

أظهرت النتائج أن هناك تزامن ايجابي في مستوى كل من البروجيستيرون progesterone والكورتيزول cortisol في المصل واللعاب في الارتفاع والانخفاض، بينما كانت النتائج غير متوافقة لهرمون الاستراديول estradiol الموجود في اللعاب.

في حالة الإناث اللواتي تم تقسيمهن استنادا الى العمر، فقد أظهرت النتائج أن هناك فرق معنوي ($P<0.05$) في مستوى الهرمونات الستيرويديه لكل من المصل واللعاب لمختلف الفئات العمرية.

أما الإناث المصنفات استنادا إلى نوع العقم، بينت النتائج أن هناك انخفاض معنوي ($P<0.05$) في معدل الهرمونات الستيرويديه في اللعاب مقارنه مع مستواها في المصل. بينما أظهرت النتائج للإناث المصنفات استنادا إلى الحالة الزوجية والخصوبيه وجود فرق معنوي ($P<0.05$) لمستوى الهرمونات الستيرويديه بين المصل واللعاب للإناث المتزوجات وغير المتزوجات، وكذلك بالنسبة للإناث العقيمات وغير العقيمات.

وعند المقارنة بين الإناث المتزوجات وغير المتزوجات، فقد أظهرت النتائج أن هناك فرق غير معنوي($P>0.05$) في مستوى الهرمونات الستيرويديه لكل من المصل

واللعاب. ولكن عند ألمقارنه بين النساء العقيمات وغير العقيمات وجد أن هناك فرق معنوي($P<0.05$) في مستوى الاستراديول estradiol والكورتيزول cortisol في المصل، وفرق غير معنوي ($P>0.05$) لهرموني الاستراديول estradiol والكورتيزول cortisol في اللعاب، أما هرمون البروجيستيرون progesterone الموجود في اللعاب والمصل فقد لوحظ عدم وجود فرق معنوي ($P>0.05$) عند المقارنة بين الإناث العقيمات وغير العقيمات.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

تقويم مستوى الهرمونات الستيرويديه للمصل واللعاب في الإناث

رسالة مقدمه إلى كلية العلوم جامعة النهرين

كجزء من متطلبات نيل درجه الماجستير في علوم التقنيات الاحيائيه

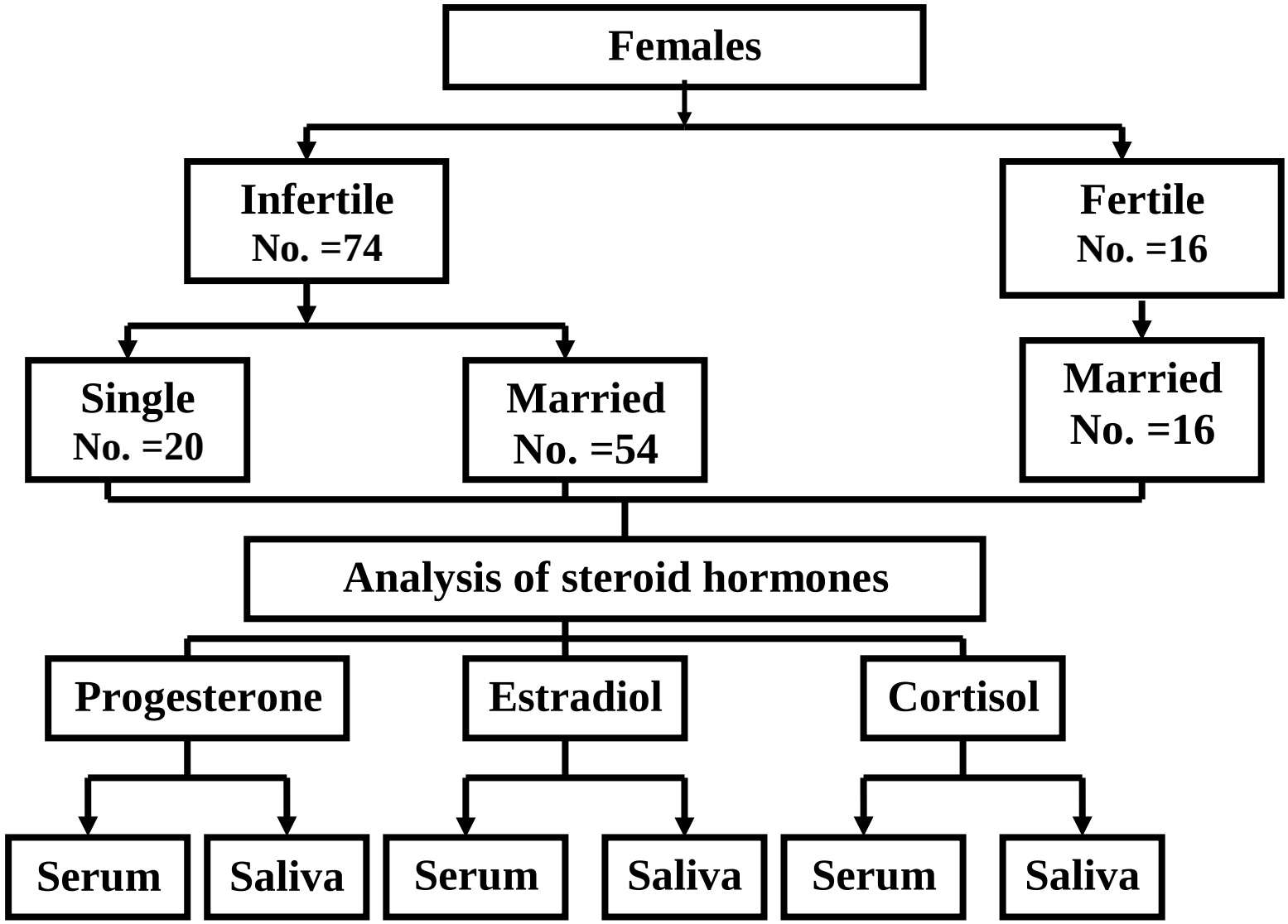
من قبل

نور عادل عبود

بكالوريوس علوم التقنيات الاحيائيه
(٢٠٠٥)

جمادي الاولى ١٤٢٨ حزيران
٢٠٠٨

Figure (2-1): scheme of experimental design



Chapter one

Introduction and Review of Literatures

Chapter two

Subjects, Materials and Methods

Chapter three

Results

Chapter four

Discussion

Conclusions and suggestions

References