The Clinical Significance of Luteal Support Therapy in the Stimulate Cycle Following the Transfer of Superovulated Embryos

Mundhir T. Ridha-Barzanchi, DVM, PhD*
Ahmed K, Allow, MBChB, PhD**
Amer S. Balafair, MBChB, PhD***
Ahmed M. Taiyeb, B.Sc, Pharm.****
Saeeda, A.M.Al-Anssari, BVMS, PhD*****

Summary:

***Background**: Ovulation induction by gonadotropin in in-vitro fertilization (IVF) program results in luteal phase defect (LPD). Luteal support therapies are considered to be important treatment to support the implantation of transferred superovulated and IVF embryos.*
***Objective**: The objective of the study was to investigate the effect luteal support protocols (LSP) on embryo implantation of 2-cell, 4-cell, and 8-cell and morulae following superovulation and embryo transfer as an animal model for human embryo transfer.*
***Materials and Methods**: Mature healthy hamsters were superovulated by human menopausal gonadotropin (hMG) and human chorionic gonadotropin (HCG). Embryo transfer was performed on day 6 of the cycle. The LSP consisted of 0.04 mg progesterone (P)/day, injected intramuscularly (I.M, protocol one) and 0.04 mg P plus 2.5 international units (I.U.) hCG/72 hours (Protocol two) and 0.04 mg P plus 2.5 IU plus 0.20 mg/ day intraperitoneal injection of aspirin. All the luteal support protocols started from day 5 to day 16 of the cycle. The animals were divided in to a control and treated groups. The control and treated groups were subdivided into subgroups according to embryo developmental stages (2-cell, 4-cell, 8-cell and morulae).*
***Results**: Superovulation (SO) caused a significant ($P<0.01$) increase in the number of morphologically abnormal embryos compared to the control group. The implantation rates of the SO embryos were significantly ($P<0.05$) decreased compared to the control group. The implantation rates of the 8-cell and morula embryos of the SO group were significantly higher than the 1-cell and 2-cell embryos in protocol's one, two and three. Significantly higher implantation rates of all the embryo stages were observed in protocol three compared to protocols two and one.*
***Conclusion**: It was concluded from the results of the study that SO markedly affected luteal function of the corpus luteum and reduced embryo implantation. Luteal support protocols particularly supplementation of progesterone with HCG and aspirin resulted in significant improvements in the implantation of 2-cell, 4-cell, 8-cell and morula embryos.*
***Keywords:** Embryo implantation; Luteal support therapy and Embryo transfer*

Introduction:

There are many factors affecting the outcome of embryo implantation and pregnancy rates in the program of in vitro fertilization and embryo transfer (IVF-ET). These factors include ovarian stimulation protocols, age of the donor and recipient, quality of the ova and embryos, levels of follicular phase estrogen, number of transferred embryos and type of luteal support therapy (1-3). Pregnant mare serum gonadotropin (PMSG) and human menopausal gonadotropin (hMG) are widely used for ovarian superovulation in IVF-ET programs.
The roles of LH in ovarian folliculogenesis are still subject to controversy (4). It is questionable whether the presence of LH activity is necessary for adequate multiple follicular development. It has been postulated by the above authors that LH administration may have an adverse effect on embryo implantation, whereas, others have not detected a detrimental effect of the exogenous LH-like activity contained in hMG (5-6). The use of hMG for ovarian stimulation was found to cause a 3 to 4 days shorter luteal phase compared to the spontaneous menstrual cycle in rhesus monkeys (7). Many authors reported that controlled ovarian stimulation impairs the function of the corpus luteum and induces luteal phase insufficiency and inhibit embryo implantation (8-10).

Stouffer et al., (11) reported that reduced luteal phase serum progesterone concentrations were correlated with the circulating human chorionic gonadotropin (hCG) disappearance. Vande-Voor et al., (12) observed that the hyperstimulated Rhesus monkey ovaries responded to exogenous HCG treatment regimen and the defects in the corpus lutum function were not due primarily to corpus luteum impaired sensitivity to gonadotropin. They concluded that defect in luteal development and

IVF Institute For Embryo Research and Infertility Treatment Baghdad University Teaching Hospital, Baghdad, Iraq
**Present Address: Reproductive Sciences Lab, Texas A&M University, College Station,*
***Head of Physiology Department, Sana'a Medical College, Sana'a University, Yemen*
****General Director of Ibin Sina Teaching Hospital, Hadramout University Medical College, Fowa, Mokalla, Hadramout, Yemen,*
*****Director of General Kanaqin Hospital Pharmacy, Kanaqin, Diyala, Iraq,*
******Director of IVF Lab. IVF Center, Baghdad University Teaching Hospital, Iraq*

abnormal gonadotropin milie, which remained after ovulation induction, may have caused changes in the lifespan and physiology of corpus luteum during the cycle and early pregnancy.

In the human, IVF treatment cycle, ovarian stimulation induces luteal defect and luteal support therapies are essential to support corpus luteum function (13). The use of exogenous progesterone (P) to overcome the effect of estrogen in IVF – ET and to support the function of the corpus luteum has been widely used in humans diagnosed with infertility. The purpose of this study was to use three protocols of luteal support therapy to support embryo implantation following superovulation and embryo transfer. The effect of superovulation on embryo morphology was also studied. Golden hamsters were used in the present study as a model for the human luteal support in IVF-ET program.

Materials and Methods

1. Animal management

Mature hamster 10 to 12 weeks old and weighing 88 to 110 grams were obtained from the animal house of IVF Institute For Embryo Research and Infertility, Baghdad Teaching Hospital, University of Baghdad. They were kept in an air-conditioned room and automatically controlled photoperiod regimen. The light period was from 6:30 A.M. to 7:30 P.M. The temperature of the animal house was maintained at 23C. Golden hamsters were housed in opaque plastic cages measuring 45 X 37 X 15 cm. Four hamsters were kept per cage and qualified veterinary specialist supplied tap water and diet. The animals were kept under observation for at least two estrous cycles for Adaptation. All the cages were cleaned and sterilized with 70% ethanol once a week regularly. Only healthy hamsters without abnormalities in their cycles were used in this study.

2. Estrous cycle

Phases of the estrous cycle were examined and reported by using vaginal smears. The smears were performed daily between 8:00 A.M. and 11:00 P.M. for at least two estrous cycles. A sterile loop was introduced into the vagina to take vaginal smear. The smear was placed and spread on a clean slide and stained with 1% methylene blue for 4 minutes. Slides were washed with tap water and dried and examined under a compound microscope (Olympus BHC microscope, Japan). The stages of the estrous cycles were checked and those hamsters, which showed regular normal estrous cycle, were involved in the present study as described previously (8).

3. Vasectomy

Fertile hamsters were anesthetized with an intraperitoneal injection (IP) of 0.045 ml of pentobarbitone sodium (Nembutal, Abbott Laboratories, 60 grams per 100 ml). Vasectomy was performed via an incision on the scrotal region. Adipose tissue and vessels were removed away from vas deferens region. The vas deference was ligated using surgical suture (Cut Gut, Ethincon LTD, Scotland) in two locations (Upper and lower). The middle area between the two ligatures was cut using sterilize scissors. The adipose tissue and blood vessels were returned to their position. Antibiotic powder was applied to the wound. The vasectomies males were housed individually for at least 24 days before use. The infertile vasectomies males were used to induce pseudopregnancy in recipient hamster as described previously (8).

4. Superovulation

Human menopausal gonadotropin (hMG, Pergonal, each ampoule contains 75 I.U FSH and LH per ampoule, Serano Company, Italy) and human chorionic gonadotropin (hCG, Pregnyl, 1500 IU, N.V.Organon, Oss Holand) were used for induction of ovulations in the treated group (Superovulated group). The dose of hMG was 20 IU administered by IP route on day one and two of the estrous cycle. The HCG was injected IP in a dose of 30 IU on cycle day 4. The superovulated hamsters were mated to vasectomies males and used as recipient animals. Naturally ovulated female hamsters were mated to vasectomies males at estrous (14).

5. Embryo recovery

The donor animals were sacrificed by manual decapicitation and embryos at different embryonic stages (2-cell, 4-cell, 8-cell and morulae) were flashed and recovered from oviducts and uterine horns. Tuberculin syringes (1ml) with 30-gauge needles containing 0.3 ml tissue culture medium (TCM-199) were used to recover the embryos from oviducts and 25-gauge needles with 1 cc tuberculin syringes were used to recover the embryos from uterine horns. The embryos were examined under a high power dissecting microscope (Wild Heerbrugg, M3 Sweden) for morphological normality. The embryos from both groups (superovulated and control groups) without abnormalities such as changes in cell volume, blastomere shape, fragmentation, crack in zona pellucida and vacuolation in the cytoplasm were selected for transfer (15).

6. Embryo transfer

The period of synchronization was the same for the donor and recipients. The recipient hamsters were prepared for embryo transfer (ET) after induction of pseudopregnancy. The hamsters in the superovulated and control group were anesthetized with pentobarbitone sodium. A dorsolateral incision through the abdominal wall at the flank region was made after shaving and sterilizing the site of operation. The embryos were injected into the right and left uterine horns by using a fine micropipette connected to a plastic mouth tube. The 2-cell embryos were injected into the oviduct through the ovarian bursa. The micropipette was checked after each embryo deposition to assure

the embryos were transferred. The animal was killed on day 16 of the cycle and the uterus was dissected and examined for implantation sites.

7. Experimental design

7.1 Experiment one

In this experiment, the effect of superovulation hormones (hMG and HCG) on embryo morphology was studied. The donor animals were divided into a superovulation
and naturally ovulated groups. Embryos were recovered from both groups and morphologically examined under a high power-dissecting microscope. The numbers of morphologically normal and abnormal embryos were recorded. The number of the animals in the control group was 38 and in the supereovulated group was 56.

7.2 Experiment two

In this experiment, the effect of recipient superovulation on implantation of 2-cell, 4-cell, 8-cell and morulae was studied. The luteal function of the corpora lutea was supported by intra-muscular (I .M.) injection of progesterone (Progestrone B.P, 25 mg ampoule, Biologici, Italia, Navate-Melano-Italy), in a dose of 0.04 mg per day from day five to day 16 of the cycle. The number of the animals was 60 in the control group and in the superovulated group.

7.3 Experiment three

The aim of this experiment was to study implantation rates of hamster 2-cell, 4-cell, and 8-cell and morula following superovulation, embryo transplantation and support of the function of the corpora lutea by a combination of progesterone and HCG treatments. The progesterone treatment was similar to experiment two plus an I.M. injection of 2.5 IU per animal of HCG every 72 hr from day 5 to day 16 of the cycle. The number of the animal per each of embryonic stage of development was 15 in the control and superovulated groups.

7.4 Experiment four

The design of this experiment was similar to design of experiment three except that the function of the corpora lutea was supported with aspirin in addition to progesterone and HCG treatments. Aspirin in a dose of 0.2 mg per day per animal (Aspegic injection, 0.5 gm, Laboratories Synthelabo/ Synthel ABO Group, France) was given I.P. from day 5 to day 16 of the cycle. The number of hamster per each group of the embryonic stage of development was 15.

8. Statistical analysis

The percentages of morphologicaly normal embryos and embryo implantations were compared between control and treatment groups and between treatments groups within each experiment were tested by analysis of variance (ANOVA) and Duncan's test using statistical analysis system (SPSS 7.5 Inc. Chicago, IL). A statistical level of 5% was considered statistically significant at $P<0.05$ (16).

9. Acknowledgment

The authors would like to thank Dr. Duane C. Kraemer, professor at Texas A and M University, College Station, Texas for review the manuscript and providing valuable suggestions.

Results:

In experiment one the effect of superovulation hormones (hMG and hCG) on embryo morphology and normality was studied and compared to a control group (Non-superovulated). Two hundred and fifty nine embryos were recovered from naturally ovulated donors with a mean of 6.81 embryos per animal. The number of recovered embryos from the superovulated donors was 748 with a mean embryo recovery rate of 13.35. The percentage of the normal embryos in the control group was 91.07 and in the superovulated group was 80.27. The percentage of abnormal embryos in the superovulated group was significantly higher than that of control group (19.37 versus 8.90, respectively, $P<0.01$, Table 1) In experiment two, the implantation rates of 2-cell, 4-cell, 8-cell and morula embryos were investigated in the naturally ovulated recipient with out luteal support and in superovulated recipients in which the function of corpora lutea was supported by IM injection of progesterone. The implantation rates of the control groups were significantly higher than the superovulated groups ($P<0.05$). The implantation of the morula stage was significantly higher than that of 2-cell and 4-cell embryos (62.58 versus 43.25 and 50.26 respectively, Table 2, $P<0.05$). The implantation rate of 8-cell embryos was also significantly higher than that of 2-cell and 4-cell embryos (60.72 versus 43.25 and 50.26 respectively, $P<0.05$, Table 2).

The results of experiment three showed similar results to those of experiment two. The implantation rates of 2-cell, 4-cell, 8-cell and morula stage in the control group were significantly higher when compared to corresponding embryonic stages in the super- ovulated group, (65.50 versus 50.04, 72.85 versus 56.35, 75.72 versus 66.57 and 80.60 versus 70.42, $P<0.05$, Table 3). The implantation rate of morula was 70.42 and the implantation of 8-cell embryos was 66.57. Both morulae and 8-cell embryonic stages had implantation rates significantly different from 2-cell and 8-cell stages in the super- ovulated group ($P<0.05$, Table 3).

The results of experiment four are shown in table 4. Luteal function of corpora lutea was supported in the recipient hamsters using progesterone plus HCG and aspirin treatment. There were improvements in the implantation rates of all embryonic stages in the superovulated groups when compared to that of experiment two and three. The implantation rates of the superovulated 2-cell, 4-cell, and 8-cell and morula stages were 47.55,

54.29, 69.38, 75.85, respectively. The 8-cell and morulae stages had significantly higher implantation rates than 2-cell and 4-cell superovulated groups ($P<0.05$, Table 4). The control 2-cell, 4-cell, 8-cell and morula developmental stages had significantly higher implantation rates then their crossponding superovulated groups. In order to find which luteal support protocol gave best improvement in embryo implantation rate, comparisons were made between the results of the three luteal support protocols in regard to the different developmental stages of the hamster embryos. The results are shown in Table 5. The most significant improvements were observed in protocol three which gives higher implantation rates in all embryonic stages studied when compared to protocol one. The implantation rates of protocol two were also significantly higher than protocol one except for the 8-cell stage, which was relatively higher but not statistically significant. The overall implantation rate in the protocol three was 61.76, and in the protocol two was 60.84, and in the protocol one was 54.20. The implantation rate of protocol three and two was significantly different from protocol one ($P<0.05$).

Table 1: The effect of superovulation hormones on embryo recovery and morphology.

Groups	Animal Number	Recovered Embryos	Recovery Rate	Percent Normal Embryos	Percent Abnormal Embryos
Control	38	259	6.81	91.07	8.93
Treated	56	748*	13.35*	80.27*	19.73*

*P<0.01 significantly different from control group

Table 2: The effect of superovulation on implantation rates of hamster preimplantation embryos following embryo transfer and application of progesterone therapy (Protocol 1, 0.04 mg progesterone/day from day 5 to day 16 of the cycle).

Groups	2-cell (%)	4-cell (%)	8-cell (%)	Morulae (%)
Control	61.46*	70.08*	73.86*	81.72*
Treated	43.25b	50.26b	60.72a	62.58a

*P<0.05 Star indicates significant difference from corresponding treated group.
Different superscripts within the same row indicate significant differences (P<0.05).

Table 3: The effect of superovulation hormones on implantation rates of hamster embryos following embryo transfer and application of progesterone and human chorionic gonadotropin (hCG) treatment to support the function of corpora lutea (0.04 mg progesterone plus 2.5 I.U hCG/day from day 5 to 16 of the cycle).

Groups	2-cell (%)	4-cell (%)	8-cell (%)	Morulae (%)
Control	65.50*	72.85*	75.72*	80.60*
Treated	50.04b	56.35b	66.57a	70.42a

*P<0.01: significantly different from corresponding treated group.
Different superscripts within the same row indicate significantly different (P<0.05).

Table 4: The effect of superovulation hormones on implantation rates of hamster embryos following embryo transfer and luteal support therapy with progesterone plus HCG and aspirin (0.2 mg aspirin/day I.P injection from day 5 to 16 of the cycle).

Groups	2-cell (%)	4-cell (%)	8-cell (%)	Morulae (%)
Control	63.96*	73.15*	74.82**	82.92**
Treated	47.55b	54.29b	69.38a	75.85a

*P<0.0 1, **P<0.05: significantly different from corresponding treated group.
P<0.05 (a versus b): different superscripts within the same row significantly different.

Table 5: The clinical significance of luteal support protocols in embryo implantation following embryo transfer in stimulated cycles.

Embryonic Stages	Protocol One (%)	Protocol Two (%)	Protocol Three (%)
2-cell	43.25a	50.04b	47.55b
4-cell	50.26a	56.35b	54.29ab
8-cell	60.72a	66.57ab	69.38b
Morulae	62.58a	70.42b	75.85b
Over all	54.20a	60.84b	61.76b

Different superscripts within each row indicate significant differences between protocols (P<0.05). The number of the hamster per each group (developmental stage) was 15.

Discussion:

The number of embryos from superovulated donors in experiment one was significantly higher and 3 fold greater than the number embryos from naturally ovulated donors (Control group), but the percentage of the morphologically normal embryos was significantly higher in the control group compared to superovulated group. Other workers reported that superovulation causes a 2 to 3-fold increase in the number of the ova ovulated (1). The increase in the number of the ova is due to the stimulatory effect of hMG on the hamster ovaries (17). Shaher (9) found that superovulation increases significantly the number of morphologically abnormal embryos in the rat. This may be due to the elevated steroid hormones and its detrimental effect on the developed follicles and cleaved embryos. It has been reported by other investigators that an increase in estradiol concentration results in reduction in oocyte quality (18). Exogenous gonadotropin treatment causes an increase in the rates of degenerated embryos and triploidy with low fertilization rates (19). It was also reported that superovulation induced a significant delay in hamster ovulation and an increase in the recovery time of superovulated embryos compared to naturally ovulated hamsters (1).

Luteal support protocols for recipients were used in experiment two, three and four in the superovulated groups but the implantation rates were significantly lower than those of control-

nonsuperovulated group. The implantation rates of the 2-cell, 4-cell, 8-cell and morula stage embryos were significantly reduced compared to their corresponding embryonic stages in the naturally ovulated embryos transferred to recipients without luteal support protocols. Marked significant reduction were observed in the 2-cell and 4-cell embryos versus 8-cell and morulae. These findings are in good agreement to those of previous workers (15) who reported in hamsters significant reduction in the implantation rate of frozen-thawed superovulated early embryonic stages versus late embryonic stages. Gosh et al., (14) also reported that after 8-cell hamster embryo transfer, the uptake of progesterone by the uterine tissue increased in naturally ovulated and superovulated pregnant hamsters compared to 2-cell hamster embryos. The increase in the progesterone uptake in the 8-cell embryo transfer group may increase 8-cell embryo implantation rate. Shaher (9) also observed in the rat that the implantation rates of the superovulated embryos transferred to superovulated pseudopregnant recipients were significantly reduced (<50%) compared to naturally ovulated donor-recipient embryo trasfer. Other workers (2) reported higher number of degenerated embryos and abnormal histophysiological structural changes in the ovaries and uteri of superovulated recipients compared to naturally ovulated recipients in rat. Fossum et al, (20) found that in the mouse, superovulation resulted in inhibition in embryo implantation. It seems that exogenous gonadotropic hormones cause abnormal increases in the steroid production particularly estradiol and this has an adverse effect on the negative feed back mechanism and results in desensitization of pituitary by reducing gonadotropin relase which reduce the implantation of the transferred embryos (12,21).

The data of experiment five demonstrate that supplementation of luteal support of the recipient females following embryo transfer in the superovulated groups resulted in significant improvements in the embryo implantation rate in protocol three compared to protocol two and one. The implantation rates of the preimplantation embryos (2-cell, 4-cell, 8-cell and morula) were also significantly higher in protocol two compared to protocol one. Progestrone is an important factor in embryo implantation and hCG indirectly supports embryo implantation through stimulation of progesterone secretions from corpus luteum (22). Chwalisz, et al., (23) found that progesterone injection improves the endometrial receptivity as well as inhibits adhesion and invasion of transferred embryos. The hCG administration for luteal support following embryo transfer causes normalization of the uterine milieu after superovulation (13). The supplementation by aspirin resulted in significant improvement in embryo implantation in the superovulated group compared to protocol one. Aspirin is a non-steroidal anti-inflammatory drug and its positive action on embryo implantation may be due to its irreversible inhibition of the cyclooxygenase-2 enzyme. This enzyme is required for conversion of thromboxan-A2 to prostaglandin E2 (PG-E2) and PG-F2a leads to improvement in corpus luteum function. PG-F2a originates in the uterus and the cells of corpus luteum and has luteolytic action on the corpus luteum, and was found also to be an inhibitor of gonadotropin-stimulated progesterone production of luteinized cells (24).

In conclusion, superovulation induces marked luteal phase defect in recipient females receiving superovulation in stimulated cycles. The combination of progesterone plus hCG and aspirin resulted in the improvement of embryo implantation rates especially following the transfer of later stage of embryos. The authors suggest that the results of the present study may be useful to be applied in human IVF-ET program

References:

1. *Ridha-Barzanchi, M.T.R, Dukelow, W.R. "The effect of ovarian stimulation hormones, transfer media, and side of transfer on implantation rates of transferred hamster embryos: model for human embryo transfer" J. Facul. Med., Baghdad. 1990, 32: 39- 46.*
2. *Shaher, N., Ridha-Barzanchi, M.T. " The effect of superovulation hormones on rat embryo implantation" Asian Symp. Anim. Biotechnol. 1996, 2: 10- 16. (China)*
3. *Kolb, B.A., Paulson, J. "The luteal phase of cycles utilizing controlled ovarian hyperstimulation and possible impact of this hyperstimulation on embryo implantation" American J. Obstet. Gyn. 1997, 176: 1262- 1269.*
4. *Strehler, E., Abt, M., El-Danasouri, I., De-Santo, M., Sterzik, K. "Impact of recombinant follicle stimulating hormone and human menopausal gonadotropin on in vitro fertilization" Fertil. Steril. 2001, 75: 332- 336.*
5. *Filicori, M. "The role of luteinizing hormone in folliculogenesis and ovulation induction" Fertil. Steril. 1999, 71: 405- 414.*
6. *Levi, A., Drews, M.R., Bergh, P. A., Miller, P.T., Scott, R. T. "Controlled ovarian hyperstimulation dose not adversely affect endometerial receptivity in IVF cycles" Fertil. Steril. 2001, 76: 670- 674.*
7. *Vande-Voort, C.A., Stouffer,R.L." Superovulation of Rhesus monkeys with exogenous gonadotropins evokes short luteal phageses" Program of the 5th World Congress on In Vitro Fertilization and Embryo Transfer, Norfolk, VA. April 5- 10, 1987, PP: 506.*
8. *Alsultani, Y.T.,Ridha- Barzanchi, M.T. " The effect of superovulation on implantation of cryopreserved mouse embryos" Iraqi J. Vet. Med. 1993, 17:195-203.*
9. *Shaher, N. "Embryo Production and Transfer in Rat" Ph.D Thesis, University of Baghdad, 1994, PP: 164- 171.*
10. *Soliman, S., Daya, S., Collins, J., Hughes, E. G. "The role of luteal support in infertility treatment : a meta analysis of randomized trials" Fertil. Steril. 1994, 53: 495- 499.*
11. *Stouffer, R.L., Hodgen, G.D., Graves, P.E., Danforth, D.R., Eyster, K.M., Ottobre, J.S. "Characterization of corpora lutea in monkey after superovulation with human menopausal gonadotropin or follicle stimulating hormone" , J. Clin. Endocrin. Metab. 1986, 62: 833.*
12. *Vande-Voort, C.A., Molskness, T., Stouffer, R. "Adenylate cyclase in the primate corpus luteum during stimulated early pregnancy: homologus versus heterologus desensitization" Endocrin., 1988,112: 734.*

13. Hutchinson-Williams, K.A., Decherney, A.H., Lavy, G., Diamond, M.P., Naftolin, F., Lunenfeld, B. "Luteal rescue in in vitro fertilization-embryo transfer" Fertil. Steril. 1990, 53: 495- 499.
14. Gosh, M., Ridha-Barzanchi, M.T., Dukelow, W.R. "In vitro uptake of estradiol and progesterone in the hamster uterus relative to the time of embryo transfer and stage of development" Steroid. 1982, 40: 133- 138.
15. Ridha-Barzanchi,M.T., Dukelow, W.R. "The developmental potential of frozen-thawed hamster preimplantation embryos following embryo transfer: viability of slowly frozen embryos following slow and rapid thawing" Anim. Reprod. Sci. 1985, 4: 237.
16. Gill, J. "Design and Analysis of Experiments in the Animal and Medical Sciences" The Iowa State University Press, Iowa, 1987.
17. Taya,K., Greenwald, G.S. "In vitro and in vivo ovarian steroidogenesis in the long term hypophysectomized hamster" Endocrin. 1980, 106: 1093- 1098.
18. Gelety, T.J., Bugalos, R.P. "The effect of high estradiol levels(5000 pg/ml) on implantation rates in IVF cycles" 41st Annual Meeting of the Pacific Coast Fertility Society. April 14-18, 1983, Abstract.
19. Sato, F., Marrs, R.P. "The effect of pregnant mare serum gonadotropin on mouse embryo fertilized in vivo or in vitro" J. In Vitro Fertil. Embr. Trans. 1986, 3: 353- 357.
20. Fossum, G.T., Davidson, A., Paulson, R.T. "Ovarian stimulation inhibits embryo implantation in the mouse" J. In Vitro Fertil. Embr. Tran. 1989, 6: 7- 10.
Martikainen, H., Ronberg, L., Ruokonen, A., Kaupilla, A "Anterior pituitary dysfunction during luteal phase following ovarian hyperstimulation" Fertil. Steril. 1987, 47: 446.
22. Alsadi, R.H. "Luteal support Therapies and Embryo Implantation in In -Vitro Fertilizatio and Embryo Transfer" Diploma Thesis, University of Baghdad. 2003, PP: 78- 95.
23. Chwalisz, K., Winterhager, E., Theinel, T., Garfeild, R.E. "Synergistic role of nitric oxide and progesterone during establishment of pregnancy in rat" 1999, Hum. Reprod. 1999: 14: 542- 552.
24. Arici, A., Behrman, H.R., Keefe, D.L. "Prostaglandin and Prostaglandin-Like Products in Reproduction: Eicosanoids, peroxides and oxygen radicals" In: Yen, S.C., Jaffe, R.B., Barbieri, R.L. (Eds.), Reproductive Endocrinology, Physiology, Pathophysiology and Clinical Management, W. B. Saunders Co., New York 1998, PP: 134-152.