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Effect of increasing gonadotropin dose on superovulatory response in aged females: A study on mice

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Abstract
The study was conducted to investigate the superovulatory response in aged female mice by adjusting gonadotropin dose at Department of Theriogenology, UVAS, Lahore, Pakistan from December 2013 to May 2014. Female BALB/c mice of different ages (4-6 weeks vs 7-9 weeks) were injected with five different concentration of eCG (0, 2.5, 5, 7.5 and 10 IU) and embryonic recovery and quality was evaluated. The embryonic recovery, was significantly high in both age groups (37.00±6.54 vs 10.20±0.96; 30.60±5.26 vs 9.40±0.92) 26.00±4.46 vs 8.60±1.32) at 7.5 IU as compared to control. In 4-6 weeks old mice, the embryonic recovery and grade I embryos at low dose (2.5 IU; 16.00±1.30 and 8.60±1.32) were comparable with high dose (5.0 IU; 17.20±3.55 and 7.20±1.11) in 7-9 weeks. This revealed injecting high dose to old females mice can improve superovulatory response considering factor of age can be nullified by adjusting the gonadotropins dose.

Keywords: Superovulation, hormones (eCG, hCG), embryonic recovery, embryonic quality, BALB/c mice

1. Introduction
The changing socio-economic and cultural pressures push the martial age at older stages. Fertility drops when females get older [1], resultantly some of the couples seeks *invitro* fertilization (IVF) as the suited option among the assisted reproductive techniques [2]. Superovulation is one of the pre-requisite for carrying out the IVF protocol, for harvesting sufficient number of oocytes, to be employed in succeeding steps for fertilization *invitro*. Moreover, oocyte serves as genetic resources, for conservation of endangered species, transgenesis and cloning by nuclear transfer [3].

Superovulation involves two steps; first growth and maturation of small follicles and second ovulation of these matured follicles. For follicular maturation exogenous gonadotropins such as Follicle Stimulating Hormone (FSH), Pregnant Mare's Serum Gonadotropin (PMSG) or Human Menopausal Gonadotropin (hMG) are being used. While Luteinizing Hormone (LH) and Human Chorionic Gonadotropin (hCG) are injected to induce ovulation [4]. It has been reported that the activity of pituitary gland, releasing FSH in response to LH-RH, is increased at the time of ovulation. Therefore, the LH-RH analogue; fertirelin acetate, can be used for follicular growth [5]. Inhibin hormone decreases the level of endogenous FSH, and vice versa endogenous FSH can also be increased by decreasing inhibin. Therefore, fertiline acetate, an LH-RH analogue (5)??? And passive immunization against inhibin [6] has been used as an alternative method for superovulation in addition to common treatment combination for superovulation in mice is of eCG/hCG [7].

The success of these protocols depends on maternal age. With advancing age, the quality of oocyte deteriorates due to increased chromosomal as well as meiotic spindle abnormalities [8], and the ovarian reserve depletes. The depleted reserves lead to decreased selection of antral follicles for ovulation resulting in decreased oocyte retrieval by superovulation protocol.

Superovulation response may be improved by adjusting the dose of gonadotropin. To investigate, superovulation response to the appropriate dose of gonadotropin for harvesting maximum response in terms of oocyte number and quality; the current study was designed using the mice model.

2. Materials and Methods

2.1 Mice Management and handling

The study was conducted at Department of Theriogenology, UVAS, Lahore, Pakistan from December 2013 to May 2014. All mice were housed on 12 h of dark and light periods, and were cared on the basis of animal protocol approved by ethical committee.

2.2 Design

In order to achieve objective of study, first superovulatory response of old females was evaluated and then effect of gonadotropin dose adjustment on embryonic quality was determined. Females of two age groups young (4-6 weeks) and adult (7-9 weeks) were injected intraperitoneally with five different doses 0, 2.5, 5.0, 7.5, 10.0 IU of equine chorionic gonadotropin (eCG; CHORONO-GEST/PMSG, Intervet, UK Ltd). Forty six to fifty hours post eCG, they were treated with 5.0 IU of human chorionic gonadotropin (hCG; IVF-C Life Sciences, Korea) and exposed individually to breeding males. Then females were checked out for vaginal plugs next morning and five plug positive from each age group were selected for embryonic evaluation. Two cell stage embryos were flushed from the oviducts 1.5 DPC (day post coitus) for embryonic count and quality assessment according to generalized criteria used for embryo grading (Timeline, Figure 1) [9]

Time Line: Embryonic grading criteria	
Grade	**Blastomere Shape and Cytoplasm**
I	All cells of equal size and shape with even and regular distribution, Even distribution of cytoplasm with translucent appearance (2 cell).
II	All cells of equal size and shape with even and regular distribution, Even distribution of cytoplasm with translucent appearance but stage of development was improper (1 cell).
III	Cells unequal in shape and size, Extrusion of cytoplasmic droplets.
IV	Degenerated, Without zona pellucida,

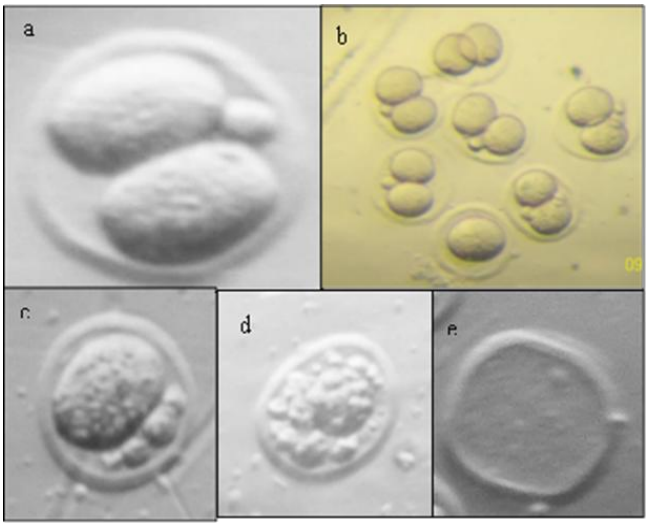

Fig 1: Grading of embryos **a,b** illustrating the normal embryos (grade 1), **b,** all other cells are at 2 cell stage but one cell at improper stage (grade II)**c,** cytoplasmic granules (grade III), **d,e,** (grade IV)**, d,** degenerated **e.** only ZP**,**

2.3 Statistical Analysis

The statistical analyses were performed using statistical software SAS Enterprise Guide (version 4.2; SAS Inst. Inc., Cary NC, USA) and statistical significance was set at $p<0.05$. Data for all parameters were presented as mean ± S.E.M. The means were separated using *Tukey's test*. One-way ANOVA was used to compare embryonic recovery at different concentration of eCG. Moreover, independent *t-test* was used to compare embryonic recovery between two age groups (4-6 weeks vs 7-9 weeks).

3. Results

3.1 Embryo recovery and morphology

In young (4-6 weeks old) females the embryonic recovery was significantly higher at 7.5 IU of eCG as compared to 0 IU (p=0.000), 2.5 IU (p=0.002), 5.0 IU (p=0.038) and 10 IU (p=0.006).

The number of transferrable embryos (grade I) were also significantly higher at 7.5 IU of eCG than 0 IU (p=0.001), 2.5 IU (p=0.008) and 10 IU (p=0.001) as shown in Table 1.

While in adult (7-9 weeks old) females, the embryonic recovery was also significantly higher at 7.5 IU of eCG than 0 IU (p=0.000), 2.5 IU (p=0.001), 5.0 IU (p=0.014). The number of transferrable embryos (grade I) was/ were significantly higher in 7.5 IU of eCG than 0 IU (p=0.022) and 2.5 IU (p=0.018 Table 1).

Table 1: Effect of dose of eCG on embryonic recovery in young and adult mice

eCG Dose (IU)	N	Embryonic Recovery		Transferrable Embryos	
		4-6 weeks	7-9 weeks	4-6 weeks	7-9 weeks
0	5	10.20 ± 0.96^{B}	9.40 ± 0.92^{A}	8.60 ± 1.32^{A}	7.60 ± 1.2^{A}
2.5	5	$16.00\pm1.30^{AB*}$	10.80 ± 1.68^{A}	$13.00\pm1.00^{AB*}$	7.20 ± 1.11^{A}
5.0	5	24.00 ± 3.86^{A}	17.20 ± 3.55^{AB}	$21.60\pm3.50^{BC*}$	12.00 ± 2.58^{AB}
7.5	5	37.00 ± 6.54^{C}	30.60 ± 5.26^{C}	26.00 ± 4.46^{C}	19.60 ± 5.24^{B}
10.0	5	19.00 ± 5.059^{AB}	25.00 ± 4.25^{BC}	8.60 ± 3.69^{a}	13.80 ± 4.59^{ab}

The interval between eCG and hCG administration was 48±2 hours and dose of hCG was (5.0 IU) in all groups, Data are presented as mean±S.E.M. n : number of plugged females, A-C represent statistical/ significant differences ($p<0.05$) within columns.*denote significant difference ($p<0.05$) within row, eCG : equine chorionic gonadotropin, hCG : human chorionic gonadotropin

When both the age groups were compared, the embryonic recovery as well as the number of transferrable embryos were significantly higher at 2.5 IU (p=0.041; p=0,005 respectively Table1).

When quality of embryos was analyzed (Table 2), then grade I embryos in 4-6 weeks at 7.5 IU of eCG were significantly higher than control (p=0.001), 2.5 IU (p= 0.008) and 10 IU (p=0.001). There was no significant difference for Grade II embryos in all treatments groups. However, grade III and grade IV embryos were significantly higher for 7.5 IU as

compared to all other doses.

While in 7-9 weeks old females, grade I embryos at 7.5 IU of eCG, were significantly higher than 2.5 IU (p= 0.018) and control (p=0.022) but non-significant than 5.0 IU (p=0.131) and 10 IU (p=0.243). Grade II embryos at 10 IU were significantly higher than only 5.0 IU (p=0.033), but non-significant difference among all other doses. There was no significant difference among all doses for grade III embryos. However, grade IV embryos were significantly higher at 10 IU as compared to other doses (Table 2).

Table 2: Effect of dose of eCG on embryonic quality

eCG Dose (IU)	N	Grade I		Grade II		Grade III		Grade IV	
		Young (4-6 weeks)	Adult (7-9 weeks)	Young (4-6 weeks)	Adult (7-9 weeks)	Young (4-6 weeks)	Adult (7-9 weeks)	Young (4-6 weeks)	Adult (7-9 weeks)
0	5	8.60 ± 1.32^{A}	7.60 ± 1.28^{A}	1.00 ± 0.54^{A}	1.60 ± 0.92^{AB}	0.00 ± 0.00^{A}	$0.00\pm.00^{A}$	0.60 ± 0.40^{A}	0.20 ± 0.20^{A}
2.5	5	13.00 ± 1.00^{AB}	7.20 ± 1.11^{A}	0.40 ± 0.40^{A}	$1.00\pm0.63^{A,B}$	$00\pm.00^{A}$	0.20 ± 0.20^{A}	2.60 ± 0.81^{A}	2.40 ± 1.02^{A}
5.0	5	21.60 ± 3.50^{BC}	12.00 ± 2.58^{AB}	0.40 ± 0.40^{A}	0.20 ± 0.20^{A}	0.20 ± 0.20^{A}	1.20 ± 0.73^{A}	1.80 ± 0.48^{A}	3.00 ± 1.14^{A}
7.5	5	26.00 ± 4.46^{C}	19.60 ± 5.24^{B}	8.00 ± 7.33^{A}	$1.75\pm0.62^{A,B}$	1.00 ± 0.40^{B}	3.25 ± 2.62^{A}	4.75 ± 2.86^{B}	3.60 ± 1.12^{A}
10.0	5	8.60 ± 3.69^{A}	13.80 ± 4.59^{AB}	9.00 ± 6.40^{A}	2.60 ± 1.02^{B}	0.00 ± 0.00^{A}	0.75 ± 0.75^{A}	1.40 ± 0.50^{A}	10.75 ± 5.58^{B}

The interval between eCG and hCG administration was 48±2 hours and dose of hCG was standard (5.0 IU) in all groups, n : number of plugged females, $^{A-C}$ denote differences within columns.*denote significant difference within row, eCG : equine chorionic gonadotropin, hCG : human chorionic gonadotropin

3.2 Correlation coefficients (r) between embryos recovered and corpora lutea

Correlation coefficients (r) between embryos recovered and corpora lutea observed (Table 3) displayed that in control group there was a significant positive correlation ($p<0.01$) between embryos recovered and corpora lutea observed (r = 0.819). However, different superovulatory concentrations were evaluated; a significant positive correlation was present among all concentrations except 10 IU. However, at 10 IU moderate positive correlation (r = 0.670) was noted.

Table3: Correlation coefficients (r) between embryos recovered and corpora lutea observed in 7-9 weeks old females mice

eCG Dose(IU)	N	4-6 weeks			7-9 weeks		
		Embryos Recovered	Corpora Lutea observed	r value	Embryos Recovered	Corpora Lutea observed	r value
0	5	10.20 ± 0.96^{b}	12.00 ± 1.44^{a}	0.819**	9.40 ± 0.92^{a}	10.20 ± 1.15^{a}	0.307
2.5	5	16.00 ± 1.30^{ab}	14.40 ± 2.35^{a}	0.976*	10.80 ± 1.68^{a}	10.80 ± 1.15^{a}	0.841
5.0	5	24.00 ± 3.86^{a}	23.00 ± 3.54^{b}	0.876**	17.20 ± 3.55^{ab}	18.80 ± 4.05^{b}	0.961^{**}
7.5	5	37.00 ± 6.54^{c}	35.40 ± 1.80^{c}	0.930**	30.60 ± 5.26^{c}	27.80 ± 2.24^{b}	0.484
10.0	5	19.00 ± 5.059^{ab}	18.40 ± 2.27^{ab}	0.670	25.00 ± 4.25^{bc}	26.20 ± 2.70^{b}	0.972^{**}

The interval between eCG and hCG was 48±2 hours and dose of hCG was standard (5.0 IU) in all groups, n : number of plugged females, $^{a-c}$ denote differences within columns. *Correlation is significant at ($p< 0.01$), ** Correlation at ($p<0.05$) significance, eCG : equine chorionic gonadotropin, hCG : human chorionic gonadotropin

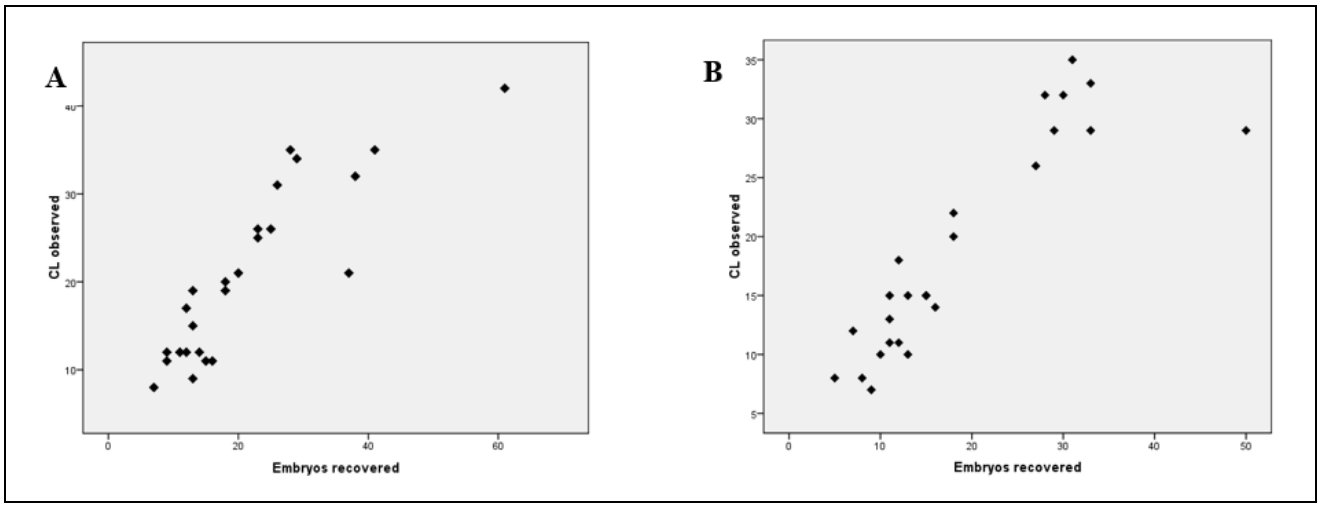

Fig 2: Correlation between embryonic recovery and CL counting (A) 4-6 weeks old female, (B) 7-9 weeks old female

Correlation coefficients (r) between embryos recovered and corpora lutea observed in 7-9 weeks (Table 3)revealed that a significant positive correlation ($p<0.01$) was at 5.0 IU (r=0. 0.961) and 10 IU (r=0. 0.972). However, a moderate positive correlation (r = 0.841) at 2.5 IU was noted. While low positive correlation was at 7.5 IU (r = 0.484) and control (r = 0.307).

4. Discussion

Superovulation is an assisted reproductive biotechnology used to obtain maximum number of oocyte from a female [10]. Gonadotropins: Pregnant Mare Serum Gonadotropin (PMSG), Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Human Chorionic Gonadotropin (hCG) are generally used. Both FSH and LH are required for *in vivo or in vitro* follicle development, whereas FSH stimulates granulosa cell growth and estradiol synthesis in certain

primary follicles. LH stimulates theca cell growth and production of androgen, resulting in ovulation of matured follicle at later stages. Female born with a finite number of ovarian cell reserves; which at each folliculogenesis depletes continuously as the life advances beyond the puberty age. As a result the fertility declines when life advances; and after a certain age in life the female require some exogenous hormonal intervention to achieve conception.

The response of superovulatory at the same dose of eCG produced more oocytes in young than adult mice. Administering 2.5 IU of eCG; 16.00±1.30 embryos were recovered from young and 10.80±1.68 from adult mice, probably due to the availability of variable ovarian reserves recruiting different number of follicles. However, comparable embryonic recovery was achieved when eCG dose was increased in adult mice than the young. In young females 16.00±1.30 embryos were recovered at 2.5 IU while in adult same number of embryos (17.20±3.55) were recovered at 5.0 IU. The literature supports the same, however at higher doses dose in old females (Table-1). This suggests injecting high dose to old females can improve superovulatory response.

The response of females of the same age to variable dose of eCG was different, owing to the fact the all follicles are equally responsive to exogenous treatments. Low dose may affect only larger follicles leading to their maturation and ovulation, whereas the high dose eCG cause growth and maturation of small follicles resulting in increased ovulations. This study confirmed that embryonic recovery between control and low dose of eCG (2.5 IU) was similar (10.20±0.96 vs 16.00±1.30), however, when 5.0 IU was injected produced significant difference (10.20±0.96 vs 24.00±3.86). The similar findings were already reported [11-17] that increasing dose of gonadotropin results in increased response.

With the increase in the dosage, a corresponding increase in response was observed in terms of embryo recovery up till 7.5 IU maximum which resulted in 37 embryos recovery at (4-6 weeks) and 30 embryos (7-9 weeks). Embryonic recoveries in both age groups were comparable to previous reports (17-20)???. However, no additional effect was found when dose higher than 10 IU, upon the number of embryos. The number rather decreased significantly in both age groups may be attributed to overstimulation of ovaries leading to either development of cyst or refractory nature of some follicles resulting in anovulation [15].

It is progressively acknowledged that overstimulation with gonadotropins result in compromised quality of developing oocyte/embryos [17]. There are several contributing factor such as hormonal imbalance resulting in alteration of uterine and oviductal environment [21] or increase in uterine thickness due to excessive estradiol production from dominant follicles [22]. This may also be due to premature leutilization of some follicle because the eCG used in this study was contaminated with LH**.** So, at the time of treatment, the follicle of ovulatory size (more than 500µm) ovulated next day afternoon before hCG injection [17] resulting in abnormal embryos development. Moreover, exogenous stimulation increases steroid production at the time of ovulation which triggered the movement of embryos from oviduct to uterine horn [17] resulted in impaired embryonic development. Long half-life of eCG which interfere with embryonic development could be another reason too [6].

In present study, the recovery of abnormal embryos increased with increasing gonadotropin dose. At lower doses (2.5, 5.0 IU of eCG) the morphologically abnormal embryos were lower and these outcomes are in agreement to the previous report [11] who investigated that degenerated oocytes were lower with low stimulation doses (10 and 15 IU FSH/LH) in the prepubertal group. However, when higher stimulatory doses were used the proportion of abnormal embryos increased. These outcomes were also reported by several other authors [6-11]. It was concluded that higher doses of the PMSG should be avoided in female of BALB/c strain to avoid abnormal embryos [11]

CL number provides an estimate for number of oocytes released. However, this method of estimating oocyte has always been criticized. It is generally reported that positive correlation exist between number of oocyte release and CL development for superovulated mice while negative for natural mice [23]. We also comes to the same conclusion in present study that positive correlation was observed between CL count and embryos recovered.

5. Conclusions

The present study revealed that superovulatory response depends upon ovarian reserves in females which are depleted with increasing age. However, increasing the dose of gonadotropins can improve embryo production in old females. But, it was observed that high dose of gonadotropin results in production of immature oocytes therefore; care must be taken while injecting high doses as high doses adversely affect embryonic quality.

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