EFFECT OF SUPEROVULATION ON OVULATORY RESPONSE, EMBRYO RECOVERY AND EMBRYO MEASUREMENTS IN BALADI RED RABBIT DOES

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ABSTRACT

Total of 24 rabbit does (5-7 mo of age, 3-4 kg LBW and 1-2 parities were used to study the effect of superovulation by PMSG on ovarian characteristics, quality and measurements of embryos at different stages (pronucli, morula and blastocyst) of Baladi Red (BR) rabbit does. Also, 3 fertile BR bucks were used for natural mating. All does and bucks were kept under the same conditions of feeding and management. Does in the 1st group (n=12) were injected with 20 mg GnRH/doe (0.2 ml Receptal) immediately after natural mating (control, G1), while does in the 2nd group (n=12) were superovulated by injection of 40 IU/kg LBW from PMSG (Foligon), followed by 0.2 ml receptal immediately after natural mating (treatment, G2). Does in G1 and G2 were sub-divided into 3 sub-groups, 4 does in each. Ovarian characteristics were determined and embryos were recovered by flushing from each treated doe in each sub-group slaughtered after 40-46 h of mating for collection of embryos at pronucli stage (1-16 cell embryos), after 60-64 h of mating for embryo collection at morula stage and after 70-72 h of mating for those at blastocyst stage. Embryos were recovered from each uterine horn and oviduct per doe by flushing and morphologically measured for thickness of mucin coat (MC), zona pellucida (ZP) and interzonal (IZ), as well as total diameter of embryos (TDE) with or without MC at different stages. Results show that average ovarian weight (right and left) and ovarian weight relative to LBW were higher (P<0.05) in G2 than in G1 (0.26 and 0.22; 0.15 vs. 0.33 and 0.27 g/doe; 0.19 g/kg LBW, respectively). Ovulatory response in terms of average number of normal follicles (large and small), hemorrhagic follicles and total follicles and average number of corpora lutea (CLs) were greater (P<0.05) in G2 than in G1 (17.75, 26.62, 0.5, 45.17 and 13.58 vs. 28.83, 32.0, 1.83, 62.67 and 22.0/doe, respectively). Ovulation rate was 74.83 and 77.15% (P>0.05) in G1 and G2, respectively. Number of embryos (viable, unviable and total) was greater (P<0.05), percentage of viable embryos was lower (P>0.05) and recovery rate of unviable embryos was higher (P<0.05) in G2 than in G1. Embryo measures including thickness of MC and ZP as well as diameter of IZ and TDE with or without MC were higher (P<0.05) in G2 than in G1, regardless embryonic stage. All embryo measures showed gradual increase (P<0.05) by increasing embryonic stage, except thickness of ZP, which showed an opposite trend (P>0.05), regardless treatment. These changes must keep in mind during cryopreservation (type of used cryoprotectants and freezing device) and embryo transfer to increase successful rates.

Keywords: Rabbit, superovulation, PMSG, ovarian characteristics, embryos.

INTRODUCTION

The rabbit is an important agricultural species and a useful model animal for biomedical research (Fan et al., 2003). Rabbit is considered as a
preferred laboratory species for the development of several reproductive technologies, such as in vitro maturation and in vitro fertilization (Chang 1959), embryonic stem cells (Graves and Moreadith 1993), transgenesis (Hammer et al., 1985) and animal cloning (Yang et al., 1985).

Superovulation is usually used in order to obtain the highest possible number of oocytes or embryos, particularly with high genetic value (Mehaisen et al., 2004). The response to superovulation treatments is highly variable and in relation with genetics, age, breeding, parity, physiological status of the animals (Takagi et al., 2001), but the hormonal preparations used are very important too. Effect of superovulation on thickness of mucin coat and zona pellucida, diameter of mass cells and embryo was reported (Fahim 1999).

Generally, superovulation may induce alteration in embryos, doing them more sensitive to cryoconservation process than non superovulated embryos (Mehaisen et al., 2005&2006). Cracked zona pellucida or mucin coat are related to suboptimal cryopreservation procedures. In several species as human or rabbits, cracked zona pellucida or mucin coat respectively is enough to reduce drastically in vivo development of cryopreserved embryos (Kasai et al., 1996). However, Viudes et al. (2010) found that in vitro developmental ability of undamaged embryos was not affected by superovulation treatment nor vitrification media in the current experiment. However, further studies of the in vivo viability of cryopreserved-superovulated rabbit embryos with dextran addition to the vitrification media must be done.

Superovulation in rabbits using FSH or eCG have been applied to increase the folliculogenesis and ovulation rate, however, may cause an increase in the number of hemorrhagic follicles or a decrease in the quality of embryos (Mehaisen et al., 2005 , 2006 and Salvetti et al., 2007). Using gonadotropines like eCG (Besenfelder, 1991) or FSH (Joly et al., 1998; Rebollar et al., 2000) was reported in rabbits. In this respect, Moor et al. (1984) indicated that eCG has a more important half life than FSH (several days versus few hours). Several authors have underlined that the use of eCG presents numerous disagreements as chromosomal abnormalities in blastocyst (Fujimoto et al 1974), alteration of ova transport in the genital tract (Adams, 1965). However, informations about the effect of superovulation in rabbit does by PMSG in the literature are scare.

Baladi Red rabbit is a local Egyptian strain which has good adaptation ability and disease resistance compared with foreign breeds and can be used as a genetic resource.

The objective of this study was to evaluate the effect of superovulation by PMSG in Baladi Red rabbit does on ovarian characteristics, quality and measurements of embryos at different stages (pronucli, morula and blastocyst stages).
MATERIALS AND METHODS

This study was carried out at the Physiology Laboratory, Animal Production Department, Faculty of Agriculture, Mansoura University.

**Animals:**
A total of 24 Baladi Red (BR) rabbit does, of approximately 5-7 months of age, 3-4 kg live body weight (LBW) and within 1st-2nd parity were used in this study as donors of embryos. In addition, 3 fertile BR bucks, around 9 months of age and averaged 3.75 kg LBW were used for natural mating.

All does and bucks were kept under the same conditions of feeding and management in a private rabbit farm. All animals were individually housed in metal cages (40 x 50 x 60 cm) provided with feeders and water in each cage. Does and bucks were fed ad. libitum on a commercial pelleted diet.

**Experimental design:**
Rabbit does in the 1st group (n=12) were injected with 20 mg GnRH/doe (0.2 ml Receptal, Intervet, Salamanca, Spain), immediately after natural mating by the fertile BR buck and were considered as a control group (G1), while does in the 2nd group (n=12) were superovulated by intramuscular injection of 40 IU/kg live body weight from PMSG (Foligon, Intervet International B.V., Boxmeer, Holland), followed by 0.2 ml GnRH analogue (Receptal) immediately after natural mating and served as treated group (G2).

Rabbit does in both control and treated groups (G1 and G2) were subdivided into 3 sub-groups, 4 does in each. Ovarian characteristics were measured and embryos were recovered by flushing from each doe in each sub-group slaughtered after 40-46 h of mating for collection of embryos at pronucli stage (1-16 cell embryos), after 60-64 h from mating for embryo collection at morula stage and after 70-72 h from mating for those at blastocyst stage.

**Experimental procedures:**

**Ovarian characteristics:**
Regardless slaughter time of embryo collection in each sub-group, pre-slaughter weight of each doe in each group was recorded and immediately after slaughtering, ovaries were removed, washed by distilled water and dried by cleaning paper. Ovaries were collected and excised, submerged in a flacon plastic tissue culture dishes (60 x 15 mm) containing saline solution at 38.5°C.

Ovarian measurements including ovarian weight (right and left ovaries), number of corpora lutea (CLs), and small (less than 1 mm in diameter), large follicles (more than 1 mm in diameter) and hemorrhagic follicles, were recorded per/doe. Then, relative ovarian weight (ROW) to LBW, total number of follicles (TNF) and ovulation rate (OR) were calculated as the following:

\[
\text{ROW} = \left( \frac{\text{ovary weight (g)}}{\text{LBW of doe (kg)}} \right) \times 100
\]

\[
\text{TNF} = \text{Number of small + large + hemorrhagic follicles}
\]

\[
\text{OR} (%) = \left( \frac{\text{Number of CLs}}{\text{number of large + hemorrhagic follicles}} \right) \times 100
\]
Embryo recovery:  
Preparation of flushing medium:  
Phosphate buffer saline (PBS) medium was prepared according to Gordon (1994) as shown in Table (1).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/l</th>
<th>Ingredient</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ 2H₂O</td>
<td>0.133</td>
<td>KH₂PO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO₄ 7H₂O</td>
<td>0.120</td>
<td>Glucose</td>
<td>1.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.0</td>
<td>Sodium pyruvate</td>
<td>0.036</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2</td>
<td>Streptomycin</td>
<td>100 mg</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>2.17</td>
<td>Sodium penicillin G</td>
<td>100,000 I.U</td>
</tr>
</tbody>
</table>

About 2 mg from bovine serum albumin (BSA) was added to one ml of PBS (2%). The prepared medium was adjusted to pH value of 7.2-7.4 using pH-meter and to osmolarity level of 280-300 mOsmol/kg using osmometer. Then, the medium was filtered by 0.22 µm millipore filter (milieux GV, millepore, Cooperation Bedford MOA).

Embryo collection:  
Embryos were recovered from each uterine horn and oviduct per doe by flushing using PBS. The flushings were collected in sterile plastic Petri dishes and embryos were washed three times with PBS, counted and evaluated for viable and un-viable embryos under a stereomicroscope at a magnification of 20–40 x.

Immediately after embryo recovery, embryos were morphologically measured for thickness of mucin coat, zona pellucida and interzonal, as well as total diameter of embryos at different stages using eye piece in micrometer.

Statistical analysis:  
Data obtained from this study were statistically analyzed using a software package (SAS, 2000). Group differences in ovarian characteristics, ovulation rate, number and recovery rate of fresh embryos were performed using T-test to evaluate the effect of hormonal treatment. However, data of embryo measurements were subjected to analysis of variance using factorial design according to the following model: \( Y_{ijk} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ijk} \) where: \( Y_{ijk} \) = observed values, \( \mu \) = overall mean, \( A_i \) = treatment, \( B_j \) = embryonic stage, \( AB_{ij} \) = Interaction due to treatment x embryonic stage and \( \varepsilon_{ijk} \) = Random error.

The significant differences among means were tested using Duncan's Multiple Range Test (1955). The percentage values were subjected to arcsine transformation before performing the analysis of variance. Means were presented after being recalculated from the transformed values to percentages.
RESULTS AND DISCUSSION

Ovarian characteristics:
Regarding slaughter time in each group, average absolute ovarian weight (right and left) and ovarian weight relative to LBW in control group (G1) and superovulated one (G2) are presented in Table (2). These results showed significant (P<0.05) differences between both groups, being higher in G2 than in G1. The significant (P<0.05) increase in absolute ovarian weights in G2 was indicated also in term of significant (P<0.05) increase in ovarian weight relative to LBW in G2 than in G1.

Table (2): Ovarian characteristics of control and superovulated does.

<table>
<thead>
<tr>
<th>Item</th>
<th>Control does</th>
<th>Superovulated does</th>
<th>Sign.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right ovaries/doe (g)</td>
<td>0.26±0.003</td>
<td>0.33±0.004</td>
<td>**</td>
</tr>
<tr>
<td>Left ovaries/doe (g)</td>
<td>0.22±0.003</td>
<td>0.27±0.004</td>
<td>**</td>
</tr>
<tr>
<td>Both ovaries/doe (g)</td>
<td>0.48±0.004</td>
<td>0.60±0.007</td>
<td>**</td>
</tr>
<tr>
<td>Relative to LBW (g/kg)</td>
<td>0.15±0.002</td>
<td>0.19±0.003</td>
<td>**</td>
</tr>
</tbody>
</table>

** Significant at P<0.01.

These results indicated that superovulation treatment by PMSG significantly (P<0.05) increased ovarian weights as compared to the control group. In agreement with the present results, El-Ratel (2008) and Fahim (2008) found that ovarian weight of rabbit does (NZW) superovulated by PMSG and GnRH were increased. Also, Fukunari et al. (1990) found that the ovarian weight of immature Japanese White rabbit does (4 months old) treated with PMSG (50 IU/doe) was significantly higher than that of untreated does after 72 h of mating. Moreover, Gosalves et al. (1994) recorded that rabbit does treated with PMSG had heavier ovaries (0.60 g) than those of does injected with the saline solution (0.18 g).

Generally, the observed increase in ovarian weight of superovulated does was associated with significant (P<0.05) increase in number of total follicles and corpora lutea on the ovarian surface of does in G2 as compared to those in G1 (Table 3). Similar findings were recorded in NZW does (Fahim, 2008) and California does (Gosalves et al., 1994).

On the other hand, ovarian weight of rabbit does was not affected by treatment with PMSG and hCG (Daader, et al., 2003), 100-150 IU PMSG (Kennelly and Foot, 1965) or 0.5 mg FSH (Younglai, 1984).

Ovulatory response:
Results in table (3) show that ovulatory response in terms of average number of normal follicles (large and small), hemorrhagic follicles and total follicles/doe were significantly (P<0.05) greater in G2 than in G1, regardless slaughter time in each group. However, ovulation rate was not significantly affected by treatment, being higher in G2 than in G1.
Table (3): Ovulatory response of control and superovulated does.

<table>
<thead>
<tr>
<th>Item</th>
<th>Control does</th>
<th>Superovulated doe</th>
<th>Sign.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovulatory response (Number /doe):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large follicles/doe</td>
<td>17.75±0.372</td>
<td>28.83±0.505</td>
<td>*</td>
</tr>
<tr>
<td>Small follicles/doe</td>
<td>26.62±0.434</td>
<td>32.00±0.522</td>
<td></td>
</tr>
<tr>
<td>Hemorrhagic follicles/doe</td>
<td>0.50±0.161</td>
<td>1.83±0.271</td>
<td>*</td>
</tr>
<tr>
<td>Large and hemorrhagic follicles/doe</td>
<td>18.25±0.392</td>
<td>28.92±0.118</td>
<td>*</td>
</tr>
<tr>
<td>Total follicles/doe</td>
<td>45.17±0.626</td>
<td>62.67±0.791</td>
<td></td>
</tr>
<tr>
<td>Corpora lutea (CLs)/doe</td>
<td>13.58±0.484</td>
<td>22.00±0.492</td>
<td>*</td>
</tr>
<tr>
<td>Ovulation rate (%)</td>
<td>74.83±3.038</td>
<td>77.15±2.771</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Significant at P<0.05. NS: Not significant.

The present total number of follicles per doe was higher than that reported by Abdel-Khaled et al. (2010), who found that number of follicles per doe was 30.4/doe in Baladi Black rabbits and 20.8/doe in NZW does.

In accordance with the results of the present study, increasing incidence of hemorrhagic follicles following superovulation treatment was recorded by Kauffman et al. (1998); Mehaisen et al. (2005) and Salvetti et al. (2007) and incidence of hemorrhagic follicles may be due to the stimulation of immature and atretic (Adams, 1982; Bourdage and Halbert, 1988).

The significant increase in number of CL in G2 than in G1 was mainly attributed to the effect of PMSG on increasing Luteinizing Hormone (LH) surge as compared to that occurred in the control does. The rabbit is a reflexively ovulating species in which sensory and neuroendocrine stimuli act together to induce a LH preovulatory surge (Dufy-Barbe et al., 1973) and determine the ovulatory response. A short mating bout including ejaculation induces genital somatosensory cues that contribute to the activation of GnRH neurons and the consequential generation of a preovulatory LH surge from the pituitary gland. Plasma LH levels start to rise within 3 min after mating and reach a plateau within 15 to 75 min (Jones et al., 1976).

The numbers of CLs were higher (P<0.05) in GnRH treated mice than controls (Ömer Coşkun et al., 2002). Similar trend was observed by El-Keraby et al. (1991), who found that increasing GnRH does from 0.2 to 0.4 ml/doe increased number of CL/NZW doe. Also, Fahim (2008) found an increase in number of CL/ doe as a result of increasing time of slaughter after mating. In rabbits, the number of CLs ranged between 7.4 to 10.3 in different rabbit breeds treated with GnRH as compared to 6.6-8.0 in the controls (El-Keraby et al., 1991).

Moreover, Mehaisen (2005) recorded a higher number of ovulation sites (15.3 and 15.9) as compared to 13.5 and 13.2 (Vicente et al., 2003) for R and V lines (synthetic breeds), respectively. On the other hand, higher number of CLs (19.2/doe) for rabbits superovulated with PMSG was reported by Lee et al. (1991).

In agreement with the present results, several authors reported a range of ovulation rate between 40.3% and 82% among different superovulated rabbit breeds (Viudes-de-Castro et al., 1995; Bolet et al., 2000; Vicente et al., 2003; Mehaisen et al., 2005).
Number and recovery rate of embryos:

Yield of embryos (viable and unviable)/doe collected from oviduct and fallopian tube, regardless slaughter time in each group, are presented in table (4). Results show that number of embryos (viable, unviable and total) was significantly (P<0.05) higher in G2 than in G1. However, the differences in recovery rate of viable and total embryos were not significant, but only recovery rate of unviable embryos was significantly (P<0.05) lower in G1 than in G2.

Table (4): Number and recovery rate of embryos in control and superovulated rabbit does.

<table>
<thead>
<tr>
<th>Item</th>
<th>Control does</th>
<th>Superovulated does</th>
<th>Sign.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of recovered embryos/doe:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viable embryos</td>
<td>10.50±0.48</td>
<td>16.17±0.66</td>
<td>*</td>
</tr>
<tr>
<td>Unviable embryos</td>
<td>0.67±0.14</td>
<td>2.17±0.27</td>
<td>*</td>
</tr>
<tr>
<td>Total</td>
<td>11.17±0.52</td>
<td>18.25±0.79</td>
<td>*</td>
</tr>
<tr>
<td>Percentage of viable embryos</td>
<td>94.0</td>
<td>88.6</td>
<td>-</td>
</tr>
<tr>
<td>Embryo recovery rate (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viable</td>
<td>77.34±2.30</td>
<td>73.47±2.44</td>
<td>NS</td>
</tr>
<tr>
<td>Unviable</td>
<td>4.89±1.07</td>
<td>9.74±1.01</td>
<td>*</td>
</tr>
<tr>
<td>Total</td>
<td>82.24±2.54</td>
<td>82.87±2.88</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Significant at P<0.05.  NS: Not significant.

As affected by superovulation treatment, percentage of viable embryos decreased to 88.6% in G2 as compared to 94.0% in the control group (G1), indicating positive effect of superovulation treatment on number of viable embryos and negative effect on its percentage and insignificant effect on its recovery.

The present results are higher than that reported by Fahim (2008) in NZW does superovulated by PMSG and GnRH. The author found that number of embryos collected after 72 h of mating was 4.35/ovary and recovery rate was 66.7%. He concluded that number of embryos was nearly related to the number of CLs as indicated in the present study.

Embryo measurements:

Effect of treatment:

The effect of superovulation treatment on embryo measurements presented in Table (5). Embryo measures including thickness of mucin coat and zona pellucida as well as diameter of interzonal and total embryo with or without mucin coat significantly (P<0.05) increased by superovulation treatment, regardless embryonic stage.

Table (5): Embryo measures as affected by superovulation protocol.

<table>
<thead>
<tr>
<th>Embryo measure</th>
<th>Control does</th>
<th>Superovulated does</th>
<th>Sign.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness (µm):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucin coat (MC)</td>
<td>41.72±0.54</td>
<td>51.42±0.43</td>
<td>*</td>
</tr>
<tr>
<td>Zona pellucida (ZP)</td>
<td>12.08±0.24</td>
<td>16.97±0.96</td>
<td>*</td>
</tr>
<tr>
<td>Diameter (µm):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrazonal (IZ)</td>
<td>132.24±0.70</td>
<td>137.12±1.40</td>
<td>*</td>
</tr>
<tr>
<td>Total without Mucin coat (TE)</td>
<td>144.22±0.65</td>
<td>154.10±0.53</td>
<td>*</td>
</tr>
<tr>
<td>Total with Mucin coat (TE MC)</td>
<td>186.04±0.87</td>
<td>205.53±0.70</td>
<td>*</td>
</tr>
</tbody>
</table>

* Significant at P<0.05.  NS: Not significant.
Effect of embryonic stage:
The effect of embryonic stage on embryo measurements presented in Table (6). Embryo measures including thickness of mucin coat as well as diameter of interzonal and total embryo with or without mucin coat showed significantly (P<0.05) gradual increase by increasing embryonic stage. However, thickness of zona pellucida showed significantly (P<0.05) an opposite trend.

In accordance with the present results, Fahim (2008) found pronounced increase in mucin coat, diameter of interzonal and total diameter of embryo, while slight decrease had occurred in thickness of zona pellucida by increasing time of embryo collection after 24 to 72 hours of mating. These changes in embryo measures may reflect pronounced developmental competence of embryos by in vivo culture within the fallopian tube of does.

<table>
<thead>
<tr>
<th>Table (6): Embryo measures as affected by embryonic stage.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic stage</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Thickness (µm):</td>
</tr>
<tr>
<td>Mucin coat (MC)</td>
</tr>
<tr>
<td>Zona pellucida (ZP)</td>
</tr>
<tr>
<td>Diameter (µm):</td>
</tr>
<tr>
<td>Intrazonal (IZ)</td>
</tr>
<tr>
<td>Total without MC</td>
</tr>
<tr>
<td>Total with MC</td>
</tr>
</tbody>
</table>

a, b and c: Means denoted within the same row with different superscripts are significantly differed at P<0.05.

In rabbits is well known the essential role of mucin coat in the embryo development and implantation (Joung et al., 2004), therefore, the cryopreservation media and procedures must avoid damages on rabbit embryo coats. Cracked zona pellucida or mucin coat are related to suboptimal cryopreservation procedures. In several species as human or rabbits, cracked zona pellucida or mucin coat respectively is enough to reduce drastically in vivo development of cryopreserved embryos (Kasai et al., 1996). Therefore, superovulation treatment reflected impact on increasing thickness of mucin coat for protecting embryos during in vitro culture or cryopreservation.

Analysis of variance revealed that the effect of interaction between treatment and embryonic stage on all embryo measures was not significant, reflecting thicker mucin coat and wider interzonal and total embryo with or without mucin coat with slight changes in thickness of zona pellucida for embryos at pronucl, morula or blastocyst stages in superovulated than in control group (Fig. 1).
CONCLUSION

It was concluded that superovulation treatment of Baladi Red rabbit does with 40 IU/kg LBW from PMSG (Foligon), followed by 0.2 ml receptal immediately after natural mating increased ovarian weights, embryo yield and resulted in pronounced effects on embryo measurements at different embryonic stages. These changes must keep in mind during cryopreservation (type of used cryoprotectants and freezing device) and embryo transfer to increase successful rates.

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