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**Induction of Increased Conception Rate and
Litter Size by Hormonal Treatment of the Ewe**

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Induction of increased conception rate and litter size
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(c) ABSTRACT

Our study was carried out in an attempt to increase the prolificacy and improve the conception rate in cycling and anestrus ewes. To accomplish this goal, we administered the ewes with a GnRH agonist analog-containing implant for induction of pituitary desensitization. We hypothesized that gonadotropin (PMSG, followed by hCG) treatment of the desensitized ewes will induce a larger, but controlled number of follicles to grow and ovulate. Our experiments were designed to determine (1) the dose of the GnRH agonist that is sufficient for induction of pituitary desensitization in ewes, (2) the kinetics of induction of pituitary desensitization by this dose of the agonist, (3) the duration of the effect elicited by this treatment, (4) the kinetics of recovery from the desensitization state, (5) the mode of gonadotropin treatment for a successful induction of multiple, controlled ovulation.

We found that a 0.33 mg implant of a slow-release preparation of buserelin was sufficient for induction of desensitization in 100% of the ewes analyzed. Major desensitization of these ewes was already established on day 3, with a more profound effect observed on day 9 after implant insertion. With the implant in place, all ewes remained desensitized for at least 43 days. Time of recovery from the desensitization state varied among ewes, ranging between 44-93 days after implant insertion. However, removal of the buserelin implants resulted in a much faster recovery from the desensitization state and a normal response of the pituitary was regained within 5 days. On the basis of these results, we established a desensitization protocol that includes insertion of a 0.33 mg buserelin implant, followed by its removal 10-12 days later.

Ewes, desensitized by the above treatment, were further used for evaluation of different protocols of gonadotropins (PMSG, followed by hCG) administration to obtain an optimal size of ovulation (2-3/ewe/cycle) in a maximal fraction of the treated ewes. Our preliminary results at this stage of the study give a good indication that sequential injections of PMSG, followed by a single administration of hCG, will lead to satisfactory results. Further experiments directed towards determination of the exact doses and timing of gonadotropins administration are now in progress.

(d) OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

The long-term objective of this study was to design a protocol of hormonal treatment, which will improve the reproduction in sheep. Specifically, we aimed at increasing litter size per delivery and elevating conception rate. For this purpose, we have proposed to administer ewes with a long-acting analog of GnRH for establishment of pituitary desensitization, followed by exogenous treatment with PMSG and hCG for induction of follicular development and ovulation. We planned to establish the dose and duration of GnRH analog treatment required to obtain pituitary desensitization, and to determine the time and dose of gonadotropins treatment that will induce an optimal number of ovulations and improve the rate of conception.

(e) BODY OF THE REPORT

(1) Introduction

Improvement of fertility rate in domestic livestock has long been the goal of agricultural researchers. Among the several approaches employed to accomplish this task, genetic selection of ewes with relatively high fertility rate, cross-breeding with sheep of genetic merit for prolificacy, as well as different hormonal treatments are included. Neither administration of PMSG nor treatment with pulsatile GnRH were satisfactory in this respect.

We have proposed a different strategy of hormonal treatment that is based on the following rationale. The pituitary gonadotropin secretion is triggered by the hypothalamic gonadotropin-releasing hormone (GnRH). The responsiveness of the pituitary to GnRH throughout the estrous cycle is regulated by the steroid-hormone milieu, as well as the self-priming effect of GnRH itself (Clarke, 1987). However, chronic treatment with exogenous GnRH or its long-acting agonists induces a desensitized state, resulting in a refractory pituitary gland to further stimulation by GnRH (Picton *et al.*, 1990). Induction of pituitary desensitization, combined with exogenous gonadotropin treatment, has been successfully used in the clinic for the stimulation of controlled superovulations in women treated in IVF programs (Droesch *et al.*, 1989).

We have employed a similar approach to improve fecundity in ewes. Specifically, we established a protocol for desensitization of the ewe's pituitary gland, followed by induction of a controlled follicular growth and ovulation by administration of exogenous gonadotropins.

(2) Materials and Methods

Sheep: (a) Asaf ewes, two years old, and at least 60 days after last delivery, were used. The sheep were kept at Moshav Nordia, Israel. (b) Two-year old White-face (Columbia-Rambovillet) were kept at the Veterinary Science Farm of the University of Wisconsin, U.S.A.

Sonography: An Aloka 500V with a 7.5 MHz endorectal transducer were used. For scanning, each ewe was placed on its back, using an inverting, restraining device, and the transducer was inserted transrectally. The follicles were characterized by using a custom-designed computerized image analysis program.

Laparoscopy: A Richard-Wolf (Model #4023.00) with a 7 mm trocar was used. The ewes were deprived of food and water for at least 12 h (usually overnight) before laparoscopy. Each ewe was placed on its back, using an inverting, restraining device. The abdominal skin was shaved and scrubbed with an antiseptic (pollyvidone-iodide) solution. A local anesthetic (lidocaine 2%) was administered subcutaneously, 5-7 cm anterior to the udder and 3-4 cm on each side of the mid-ventral line (linea alba), the sites of the trocar insertion. The trocar-cannula device and the telescope were immersed in a solution of 70% alcohol and returned to this solution between each laparoscopy. The 7 mm trocar and cannula were inserted into the peritoneal cavity left to the midline. The peritoneal cavity was then inflated with CO₂ through the cannula that was connected to a gas cylinder. The 7 mm trocar was removed from the cannula and replaced by the telescope. After visualization of the abdominal cavity, the 5 mm trocar and cannula were introduced right to the midline. A manipulation probe was used for location of the ovaries and for counting the follicles and corpora lutea. After the trocars were withdrawn, antibiotics were sprayed at the penetration sites.

Buserelin Administration: Buserelin implants (Hoechst AG, Frankfurt, Germany), 1 cm long rods containing 3.3 mg, were used. When indicated, these 1 cm rods were cut by a razor blade into 2, 4 or 10 equal pieces containing the equivalent of 1.65, 0.825 or 0.33 mg of

buserelin, respectively. When indicated, a 1 mm buserelin implant was inserted into a 1 cm-long silastic tube (Dow Corning; Catalog #602-175] that was preimmersed in Xylene and air-dried thereafter. The buserelin pellets were implanted subcutaneously in the ear pinna of the ewes.

GnRH Stimulation Test: Three ml of blood samples were withdrawn from the jugular vein of the ewes. GnRH (37 µg/ml/ewe) was administered intramuscularly, and an additional blood sample was withdrawn thereafter at the specified time-points. The blood samples were kept on ice until separation and the serum was stored at -20°C.

Determination of Serum LH Levels: Ovine LH 5-26 (2.5 µg/25 µl of 0.5M phosphate buffer, pH 7.5) was labeled with 1 mCi of Na¹²⁵I (10 µl) by adding 10 µl of Chloramine T (1 mg/ml) for one minute. The reaction was terminated by adding 20 µl of sodium metabisulfite (1 mg/ml). The labeled hormone was eluted from a Sephadex G-50 column (0.9x20 cm) with phosphate-buffered saline (PBS, 0.05M, pH 7.5). For radioimmunoassay (RIA) determination of serum LH (50 µl and 200 µl samples), we used a β-OLH antiserum produced in our laboratory. The reaction was carried out in a volume of 0.5 ml for 48 h at 4°C, followed by addition of goat anti-rabbit γ-globulin (GARGG, 0.2 ml). Following an overnight incubation, 1 ml of cold PBS was added and the samples were centrifuged (4000 rpm, 4°C, 30 min) and the supernatant aspirated. The pellet was counted in a Kontron γ-counter. Standard curves were constructed from tubes containing 62.5-6250 pg of OLH-S26. The interassay variations were less than 10%.

Determination of Serum Buserelin Levels: Buserelin, 2 µg/25 µl of 0.5M phosphate buffer (pH 7.5), was reacted with 1 mCi of Na¹²⁵I (10 µl) by adding 10 µl of Chloramine T (0.5 mg/ml) for 1 min. The reaction was terminated by adding sodium metabisulfite (20 µl from 0.5 mg/ml), followed by 3 ml of 10 mM acetic acid supplemented with 1% bovine serum albumin (BSA). Elution was carried out on a Sephadex G-25 column (4x35 cm) with 10 mM acetic acid. Fractions of 4 ml were collected in tubes that contained 0.2 ml of 1% BSA. For RIA determination of serum buserelin levels in the buserelin-implanted ewes, we used an antiserum that was developed in our laboratory. This RIA system can detect concentrations of 5

to 200 pg buserelin/tube. GnRH (up to 200 ng/tube) does not compete for buserelin binding. The reaction was carried out in borosilicated glass tubes, containing 0.2 ml of serum samples and 0.3 ml of the antiserum and the labeled peptide. Standard curve tubes contained 0.2 ml of normal sheep serum. After 24 h of incubation at 4°C, 0.2 ml of goat anti-rabbit γ -globulin was added to each tube. The protocol further continued as described for the RIA of O-LH.

(3) Results and Discussion

It has been demonstrated by others and also confirmed by us that treatment of ewes with continuous administration of a GnRH agonist analog results in pituitary refractoriness to GnRH. Our experiments were designed at further determination of: (1) the dose of the GnRH agonist that is sufficient for induction of pituitary desensitization in ewes; (2) the kinetics of induction of pituitary desensitization by this dose of the agonist; (3) the duration of the effect elicited by this treatment; (4) the kinetics of recovery from the desensitization state; (5) the mode of gonadotropin treatment for induction of multiple, controlled ovulation.

Different doses (3.3, 1.65, 0.825 and 0.33 mg) of a slow-release preparation of buserelin (Hoechst A.G., Frankfurt, Germany) were implanted subcutaneously into the ears of 4 groups of ewes (10-15 ewes/group). Pituitary desensitization was analyzed by determination of serum LH levels before and at different time-points (20, 40, 60 and 120 min) after GnRH administration (37 μ g/ewe, i.m.). A control group of ewes, without a buserelin implant, was also analyzed. Since a maximal response to the GnRH challenge in the control group was observed at 60 min after injection (32-fold increase), this time-point was selected for routine desensitization tests. Ewes that responded to the GnRH challenge by elevation of their serum LH levels by less than two folds were considered desensitized.

We found that a 0.33 mg implant was sufficient for induction of desensitization in 100% of the ewes analyzed. Major desensitization of these ewes was already established on day 3, with a more profound effect on day 9 after implant insertion (Fig. 1). With the implant in place, all ewes remained desensitized for at least 43 days. Recovery from the desensitization state was detected in 10% of these ewes on day 44, in 80% on day 61, with 100% recovery rate obtained on day 93. In order to limit the desensitization state to a shorter time-period, implants were

removed two weeks after their insertion. Removal of implants resulted in gradual recovery over 5-6 days.

However, due to its small size (1 mm), removal of the 0.33 mg implant is hardly possible. Another set of experiments searched for a convenient method for retrieval of the buserelin implant. For this purpose, the implant (0.33 mg) was encased in 1 cm long silastic tubing. This device was then introduced subcutaneously in the ear pinna. This study utilized 3 treatments: control, covered pellets and buserelin implants inserted without a silastic tube (uncovered). Desensitization of pituitary function was evaluated by challenging with GnRH at 3-day intervals to day 18 post-implantation and to day 15 after implant removal. The study was initially performed in anestrus ewes (4 ewes/group) and later repeated utilizing cycling ewes (6 ewes/group). Results of these studies demonstrated that covered implants are as efficient as uncovered and much easier to use. Furthermore, times required for desensitization and recovery are generally similar in anestrus and cycling ewes.

Serum buserelin levels were determined in the ewes treated with the different doses of buserelin implants, using antibodies for the analog that were generated in our laboratory. Minimal level of detectability of this assay is 15 pg/ml. As expected, we found that the size of the implant was clearly reflected by the serum buserelin levels in the different experimental groups (Fig. 2). Ewes carrying the 0.33 mg implant showed serum levels of 47 ± 3.2 pg/ml buserelin on day 3 post-implantation and 18 ± 1.6 pg/ml on day 9. It is interesting to note that, in these ewes, serum buserelin concentrations after day 9 of implantation were undetectable (lower than 15 pg/ml) with desensitization state that lasted for at least an additional month. These observations demonstrate that very low serum buserelin levels are sufficient for maintaining the desensitization state.

Our results at this stage of the study indicated that a 0.33 mg buserelin implant induces desensitization in 100% of the treated ewes within 3 days, and that normal response of the pituitary is regained by 5 days after implant removal. Having established this desensitization protocol, we initiated our attempts to induce ovulation in desensitized ewes (10-12 days of inserted buserelin implant) by administration of gonadotropins starting on the day of implant

removal. A poor ovulatory response was obtained in the different groups of cycling ewes (10 ewes/group) administered with the following doses of PMSG: 600, 900 and 1200 IU/ewe, followed by one dose (1000 IU/ewe) of human chorionic gonadotropin (hCG) at either of the following time-intervals: 30, 40, 50, 60 and 70 h, after PMSG. Ovulation rate was determined by laparoscopic examination of the ovary for corpora lutea formation five days after hCG administration. A total of 150 ewes was used for this series of experiments.

It has been demonstrated recently (Fry *et al.*, 1988) that hypophysectomized ewes can be induced to ovulate by sequential PMSG administration followed by hCG. The beneficial effect of the continuous presence of FSH activity on ovulation has also been suggested in a series of studies done using a buserelin-desensitized ewe model (reviewed by McNeilly *et al.*, 1991). On the basis of these reports, we have modified the experimental protocol as follows: Desensitized, cycling ewes (10 ewes/group) were administered with sequential injections of increasing doses of PMSG (either 200 IU followed by 400 or 400 IU followed by 800 IU) at a 24 h interval, starting on day 11 after implant insertion. hCG (1000 IU) was administered 32 h after the last PMSG injection. In the first group of ewes, a 60% ovulation rate was observed by laparoscopic examination, with an average of 1 corpus luteum for each ovulating ewe. In the second group (higher PMSG doses), 75% of the ewes ovulated with an average of 2.8 corpora lutea for each ovulating ewe.

This last protocol was repeated at the University of Wisconsin on 12 desensitized cycling ewes treated with the higher (400 and 800 IU) PMSG doses followed by hCG (1000 IU). Four ewes served as a control group. Laparoscopy was performed on the day of hCG administration and repeated 6 days later. In addition, the ewes were scanned daily by ultrasound. Examinations were videotaped and an image of a selected follicle on successive days was digitized (in collaboration with Drs. R.A. Pierson and G.P. Adams, University of Saskatchewan, Saskatoon, Canada). Changes in individual follicles were characterized using a custom-designed computerized image-analysis program that was developed for ultrasound images. A map of pixel intensity was generated along a line which traversed the maximum diameter of the follicle including the antrum and the stroma-follicle wall interface. Serial maps were concatenated to

form a 3-dimensional profile from the day of first detection of the follicle to one day after ovulation.

Two examples of the general pattern of follicular response to the hormonal treatment are shown in Figs. 3 and 4. In each case, the data for the right ovary (RO) are in the top panel, and the data for the left ovary (LO) are in the bottom panel. In ewe D36 (Fig. 3), 2 follicles developed in the RO and 2 corpora lutea (CL) were identified by laparoscopy; in the LO, 2 follicles began to develop, but only one grew to ovulation-size and ovulated. In ewe D47 (Fig. 4), 3 follicles developed in the RO and 4 were recorded in the LO; laparoscopic examination in this ewe revealed 2 CL and one luteinized follicle in the RO, and 7 CL in the LO. As we were performing the ultrasound examinations, we had some questions about the timing of ovulation. This was clarified by data from image analysis (see below) and confirmed by the observations during laparoscopy.

Examples of the concatenated 3-dimensional images that resulted from our preliminary computerized image-analysis of these results are shown in Figs. 5 and 6. In each example, the axis from zero to the right represents the line across the maximum diameter of each image analyzed, and the axis from zero to the left represents serial maps as the follicle grew. The third dimension, height, represents pixel intensity; in the pseudocolors applied, red represents least intensity and white represents greatest intensity. The general growth pattern of a follicle in the LO of ewe D47 is shown in Fig. 5, where the images start with a 2 mm follicle and culminate with a 15 mm follicle near the time of ovulation. Note that the follicle wall remains narrow and demonstrates high pixel intensity. The additional feature shown in Fig. 6 (from the LO of ewe D36) is that the follicle wall changes abruptly from a narrow structure with high pixel intensity to a much broader structure with lower pixel intensity (10 on left axis) when ovulation and the associated luteinization occurred. Thus, this approach offers a much more distinct, and quantifiable evaluation of the developmental events leading to ovulation than would otherwise be possible by subjective visual analysis of the ultrasound images.

Figures 3-6 clearly demonstrate that ovulation did not occur within 24 h after hCG administration. Moreover, follicular growth continued after hCG administration for at least four

additional days. Therefore, as a consequence of these latter results, we have modified the protocol of hormonal treatment. On day 12 after buserelin implantation, the ewes were administered with 800 I.U. of PMSG, followed by additional 400 I.U. twice on days 13 and 15. The buserelin pellet was removed and hCG (1000 I.U.) was administered on the late afternoon of day 19. Laparoscopy was carried out on the morning of day 21 (average of 42 h post-hCG administration). Seven out of eleven ewes ovulated with an average of 2.0 c.l./ovulating ewe. In addition, 21 Graafian follicles/treated ewe were also demonstrated.

These results suggest that we are in the process of paving the way for the establishment of a protocol for induction of an ovulation rate of 2-3 follicles/cycle by hormonal treatment. Ovarian ultrasonography, combined with computerized image-analysis that has been found to offer a highly reliable method for evaluation of the dynamics of follicular development in the ewe, will assist in our future experiments in determination of the protocol of treatment with the optimal size of follicle development and ovulation.

(f) DESCRIPTION OF COOPERATION

Since the Israeli investigators and the U.S. collaborator used different ewe strains, most of the experiments were performed in parallel by the two teams. This experimental design allowed us to extend our conclusions to more than one single strain of ewes. The following items were taken care of specifically by either the Israeli investigators or the U.S. collaborator: The serum samples, collected by the U.S. collaborator, were hand-delivered to Israel on dry ice and all hormone assays (LH and buserelin) were performed at the Weizmann Institute of Science. The design of the buserelin implant encased in silastic tubing and initial experiments using this device were performed in the U.S. Ultrasonography analysis was performed by the U.S. collaborator.

Experimental planning and evaluation of the results were carried out on a daily basis via Bitnet and Fax. Towards the end of the second (and last) year of this project, the U.S. collaborator visited the Israeli investigators. In addition to comprehensive discussion, this trip was used for demonstration and instruction of the Israeli team in using the ultrasonography technique in the ewes.

(g) EVALUATION OF THE RESEARCH ACHIEVEMENTS

The original research proposal was planned to be executed during a period of three years. However, the funding for this project was approved for 2 years only. This fact, together with the Iraq war during January-March 1991, limited our possibilities to accomplish our experimental plans in full.

Nevertheless, many of the original research goals were accomplished as follows: (a) we have demonstrated that complete desensitization of ewes to GnRH is accomplished within 9 days after implantation of a slow-release preparation of a GnRH analog (buserelin); (b) we have shown that a dose as low as 0.33 mg (10% of the dose required to desensitize a woman) is sufficient for desensitization of the ewe; (c) we have developed a device for convenient removal of the buserelin implant; (d) we have found that recovery from the desensitization state after implant removal is rapid (within 5 days) and is accompanied by a normal response of the ewe to a GnRH challenge; (e) using ultrasonography and laparoscopy, we have developed methods for monitoring follicular development and ovulation; (f) we have succeeded in establishing a preliminary protocol using a sequential PMSG treatment, followed by a single administration of hCG that yields an average of 2.0 CL/ovulating ewe. These results suggest that the establishment of a protocol for a controlled multiple ovulation is feasible and further research is now required for its completion.

Once the protocol for the gonadotropin treatment is established, it should be followed by determination of the appropriate timing of insemination of the treated ewes. In addition, the possible requirements for progesterone supplementation, to support pregnancy at its early stages, should be evaluated. Finally, a large-scale field experiment should be performed for confirmation of the results obtained at each individual step of this study. It is our hope that financial support for the completion of this study will be made available by BARD.

(h) LIST OF PUBLICATIONS

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LH response to GnRH administration
(37 μ g of GnRH; I.M.; n=6)

a. Preimplantation state

b. Day 3 after buserelin implantation

c. Day 9 after buserelin implantation

d. Day 3 after removal of buserelin implant

Time (min) after
GnRH administration

Fig. 1

release of buserelin from implant

Fig. 2

EFFECTS OF PMSG/HCG ON FOLLICULAR DEVELOPMENT

EFFECTS OF PMSG/HCG ON FOLLICULAR DEVELOPMENT

Fig. 4

Fig. 3

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Fig. 5

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Fig. 6