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Analysis of superovulation in the hamster : 1962-1978

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Summary. This paper summarizes research dealing with the induction of spontaneous superovulation in the cyclic hamster by single injections of PMSG. Four principal approaches have been used : 1) ovulation rate as an endpoint ; 2) number and size of follicles, and staging of follicular development in serially sectioned ovaries ; 3) determining the effects of an antiserum to PMSG ; 4) measuring steroid and peptide hormone levels in normal and PMSG-treated animals. Collectively, the results show the advantages of using the hamster for studies of follicular growth and atresia.

Introduction.

For the past 16 years I have been exploring various aspects of superovulation in the hamster by single injections of pregnant mare serum gonadotrophin (PMSG). This research has been part of a larger program, devoted to the basic question, « What factors determine whether one follicle remains quiescent, another begins to develop but becomes atretic, while still a third matures and ovulates ? » (Greenwald, 1972).

Several features of the estrous cycle of the hamster have made it an ideal species for these studies : 1) the extremely regular 4 day cycle (day 1 = day of ovulation ; day 4 = proestrus) ; 2) the conspicuous vaginal discharge on the morning of day 1 which facilitates the monitoring of the reproductive status of animals in a large colony ; 3) the ease with which spontaneous superovulation can be induced in the adult hamster by PMSG contrary to the situation in the mature, cyclic rat (Welschen and Rutte, 1971).

Results and discussion.

Injection of graded doses of PMSG on day 1 of the hamster cycle results in a dose-dependent increase in the number of ovulations, with a plateau of 70 ovulations reached with 30 or 60 IU of the hormone (Greenwald, 1962). In subsequent studies, an ovulation rate of 70 ova with 30 IU PMSG has been rarely obtained which may be attributable to either strain differences in the hamsters or to seasonal variations in

ovarian responsiveness. The latter seems to be the most likely explanation since the maximal ovulatory response to 30 IU PMSG occurs in June and November to January, coinciding, in general, with the highest rates of ovulation in control animals (Moore and Greenwald, 1964*a*).

If the single injection of 30 IU PMSG is shifted to other days of the 4 day cycle, total cycle length is increased and there is a reduced ovulation of 40 to 50 eggs (Greenwald, 1962). The decreased ovulation rate coincides with a decline in the number of follicles larger than 267 μm as atresia disrupts the development of approximately 10 follicles per ovary. Hence, the optimal number of ovulations is elicited when PMSG is administered when endogenous levels of FSH are maximal (Bast and Greenwald, 1974) and when the follicles destined to ovulate 4 days later are set aside.

Based on these results a series of models were constructed to explain how PMSG affects the hamster ovary (Greenwald, 1962). It was postulated that the follicular population is divided into two groups : the *developing follicles*, approximately 10 per ovary, constitute the group maturing under the influence of endogenous gonadotrophins ; and the *reserve follicles*, a maximum of 25 follicles per ovary are recruited prematurely into the rank of antral follicles under the influence of PMSG.

In retrospect, classification of follicles based on size *per se* is not as meaningful as relating their growth to developmental stage. Accordingly, in a more recent study the development of advanced secondary and tertiary follicles was described in terms of 6 arbitrary stages (Greenwald, 1974). On this basis, the largest follicles present on the afternoon of day 1 are at stages 4 and 5 ; i.e., preantral follicles with 8 or more layers of granulosa cells or preantral follicles with about 12 layers of granulosa cells and incipient formation of the antral cavity. The number of these developing follicles on days 1 and 2 is about 14 per ovary but on days 3 and 4, the follicles descended from this group are reduced to 6-7 per ovary. It takes about 20 days before a preantral follicle with 2 to 3 layers of granulosa cells (stage 1) is able to ovulate (Chiras and Greenwald, 1977).

The identity of the reserve follicles is clarified in a recent study (Chiras and Greenwald, 1979). Following the injection of 30 IU PMSG at 0900 h of day 1, stage 2 follicles (preantral follicles with 4-5 layers of granulosa cells) correspond to the reserve follicles and begin to increase in size within 4 hrs. Normally a follicle entering stage 2 takes 12 days before ovulating (Chiras and Greenwald, 1977), whereas in PMSG-treated ovaries, the reserve follicles are transformed into antral stages (stage 6) in 28 hrs (Chiras and Greenwald, 1979). In several published (Chiras and Greenwald, 1978) and unpublished studies, I have been impressed with the rapidity with which follicles can be mobilized in the cyclic hamster.

One of the questions raised by the initial studies on superovulation was whether PMSG is needed only for the recruitment of reserve follicles or whether sustained follicular development requires the continued presence of the hormone. The question was answered by injecting ip an anti-serum to PMSG at various intervals after the sc injection of PMSG on day 1 of the cycle (Greenwald, 1963, 1973*a*, *b*). In essence, the experiments show that high circulating titers of PMSG are required continually ; ortherwise, atresia eliminates the reserve follicles.

A similar conclusion has been reached in experiments involving hamsters hypophysectomized for a week and then injected for 4 days with ovine FSH (NIH-S7)

followed by an ovulatory injection of 10 μg LH (Moore and Greenwald, 1964*b*). Under these circumstances, pretreatment with 200 μg FSH daily results in the ovulation of 32 ova, whereas a priming injection of 200 μg FSH and thereafter 50 μg FSH for 3 days causes the ovulation of only 9 ova. Thus, an initially large dose of FSH is required to mobilize follicles and subsequent levels of FSH determine whether normal ovulation or superovulation will occur. The hormonal profile of FSH in the normal cyclic hamster confirms this interpretation (Bast and Greenwald, 1974). Parenthetically, Dr. Moore and I attempted to repeat our experiments with hypophysectomized hamsters with other batches of NIH-FSH but were unsuccessful. The animals failed to develop antral follicles, evidently because of insufficient LH contamination in the preparations. Daily injection of 200 μg of NIH-FSH develops antral follicles in hypophysectomized mice and rats but not in hamsters.

Although the injection of anti-PMSG serum in intact PMSG-primed hamsters interferes with the further development of the reserve follicles, the animals still ovulate 10-12 ova : the number characteristic of the species. This gave rise to the heretical notion that the developing follicles are maintained by endogenous gonadotrophins while the reserve follicles are regulated by circulating levels of PMSG and that the two sets of follicles maintain separate identities. According to this notion, after PMSG-antiserum the ovulating follicles represent exclusively the developing group but this is strictly an assumption, Several years ago we tried to label the reserve follicles in PMSG-treated ovaries via the fluorescence antibody technique but the label was restricted to blood vessels ! However, commercially available preparations of PMSG are very crude and contain a number of nonspecific antigens. It would be interesting, for several reasons, to repeat the labelling experiments with PMSG, using more vigorously prepared « pure » hormone as the starting point to generate PMSG-antiserum.

The manuscript submitted in 1973 to Biology of Reproduction dealt with the effects of PMSG antiserum on follicular development in intact or hypophysectomized hamsters which had been injected previously with PMSG (Greenwald, 1973*b*). One of the reviewers of the paper had serious reservations about the significance of the research. However, the use of hypophysectomized hamsters with this experimental design provides an excellent way of inducing atresia in a synchonized population of large antral follicles. Bill (1978) has found that serum estradiol in hypophysectomized hamsters, given 30 IU PMSG 3 days previously, drops from 1 021 pg/ml to 461 pg/ml within one hour after administering anti-PMSG serum and by the next morning the hormone is undetectable. Moreover, the morphological changes associated with atresia induced by PMSG-antiserum closely parallel those observed when the process is reconstructed from the ovaries of normal, intact mammals in which atresia is a random, unpredictable event. Hence, the animal model opens new vistas in exploring the biochemical and steroidogenic changes associated with follicular atresia with the obvious advantages of a preparation with large numbers of mature follicles and with a definite starting point to which temporal changes can be related.

One of the questions frequently raised is : what effect does the accelerated development of the reserve follicles have on the maturation of the follicle and the subsequent course of gestation ? After 30 IU PMSG and mating, on days 2 and 3 of pregnancy 90 p. 100 of the ova recovered from the oviducts are fertilized (Greenwald, 1976). On day 8 of pregnancy, there are 29 embryonic swellings per hamster with the number

reduced to an average of 20.8 fetuses on day 16 — the day of parturition. The corpora lutea (CL) develop at the normal rate and, in fact, serum levels of progesterone are 2-4 times higher than in control animals reflecting the presence of 45 CL per animal in the PMSG-treated group. Thus, the premature recruitment of the reserve follicles by PMSG does not interfere with the viability of the ovum nor with the subsequent differentiation of the postovulatory follicle into « mature » CL. An earlier excursion into mating the PMSG-treated hamster (60 IU PMSG) led to some interesting finding, depending on when the females were caged with males (Greenwald, 1963). If isolated from males, the females ovulated an average of 60 ova, whereas if placed with males on the morning of day 2 none of the animals ovulated but instead became pseudopregnant. Subsequently it was observed after PMSG treatment that lordosis could be elicited on day 2 and that the next morning spermatozoa were present in the vaginal lavage. These findings suggest heightened and atypical levels of progesterone after high levels of PMSG. We have now found — 15 years later — that serum progesterone measured by RIA is doubled or tripled on the afternoon of day 2 by injection of 15 or 30 IU of PMSG at 0900 h of day 1 (Greenwald, Baranczuk, Connor, unpublished).

Steroid and peptide hormone levels in normal and PMSG treated cyclic hamsters has been a recurring topic for investigation. The cyclic hamster has extraordinarily elevated circulating levels of estradiol ; e.g., proestrous peak values of 150-200 pg/ml (Baranczuk and Greenwald, 1973*a* ; Saidapur and Greenwald, 1978). In fact, these levels are more closely related to the values in primates than to other species of mammals. As might be anticipated, the PMSG treated hamster shows an even further accentuation of high estrogen levels, depending on the number of additional follicles that have been recruited ; e.g., after 30 IU PMSG on day 1, serum estradiol on the afternoon of day 3 is 756 pg/ml (Baranczuk and Greenwald, 1973*b*).

We have recently measured serum levels of FSH and LH after PMSG-treatment by radioimmunoassay which has been handicapped by the cross reactivity of all three hormone — especially PMSG and LH (Greenwald, Baranczuk and Connor, unpublished). The results indicate that levels of FSH and LH are significantly reduced by day 2 presumably in response to the high steroid levels. However, following 5 to 15 IU PMSG on day 1, a definite — albeit reduced — LH surge occurs on day 4.

This brings us back to the point raised at the beginning of this paper : the ability to induce spontaneous superovulation in the cyclic hamster but not in the cyclic rat. The rat ovary *does* respond to 50 IU PMSG by developing 30-40 antral follicles but few of the treated animals ovulate and if they do, only a few eggs are ovulated (Welschen and Rutte, 1971). The cyclic hamster with its normally high levels of estrogen can tolerate even further increases in estrogen without affecting the ability of the hypothalamic-pituitary axis to release the ovulatory quota of LH at the end of the cycle. In contrast, the PMSG-treated rat seems incapable of secreting spontaneously a preovulatory surge of LH and thus the large number of follicles matured are doomed to atresia.

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Résumé. La durée totale de la croissance du follicule depuis le stade 1, caractérisé par 2 ou 3 couches de cellules folliculaires, jusqu'à l'ovulation, est de 20 jours.

L'injection de doses croissantes de PMSG le jour 1 du cycle conduit à un nombre

d'ovulations de plus en plus élevé jusqu'à un plateau de 70. Il existe normalement une population de 10 follicules à antrum en développement par ovaire et une réserve de 25 follicules préantraux qui peuvent être mobilisés par PMSG. Normalement, la croissance d'un follicule du stade 2 (4 à 5 assises de cellules folliculeuses) jusqu'à l'ovulation, demande 12 jours. Sous l'action de PMSG les follicules au stade 2 passent au stade 6 préovulatoire en 28 h et ovulent 4 jours plus tard. Les follicules ainsi différenciés ne demeurent sains jusqu'à l'ovulation que si le support gonadotrope fourni par PMSG demeure en permanence.

L'injection de PMSG stimule la stéroïdogenèse et entraîne un niveau élevé de progestérone susceptible de déclencher le comportement sexuel (lordose) dès le second jour : si l'accouplement est permis, l'ovulation n'a pas lieu et il s'établit une pseudogestation.

Les ovocytes provenant de ces follicules dont la croissance a été accélérée par PMSG sont fécondables et se développent jusqu'au terme. La sécrétion de progestérone par les corps jaunes résultant des ovulations est normale. La même technique d'injection de PMSG ne conduit pas à une superovulation chez la ratte, bien que 30 à 40 follicules se différencient. mais la décharge endogène de LH n'a pas lieu, peut-être par suite d'un niveau trop élevé de stéroïdes. Rien de tel ne se produit chez le hamster, la décharge endogène de LH n'étant que faiblement réduite.

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