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OVA-IMPLANTATION, EMBRYONIC SURVIVAL AND EMBRYONIC SPACING IN OVARIECTOMISED MICE AFTER PROGESTERONE AND OESTROGEN TREATMENT

A thesis presented in partial fulfilment of the requirements

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GENERAL INTRODUCTION

This investigation is concerned with the ovarian hormonal requirements for ova-implantation, post-implantation survival, and the spacing, of the developing young within the uterus of the ovariectomised pregnant mouse. Most attention is directed towards establishing the effectiveness of the ovarian hormone progesterone in maintaining pregnancy in mice ovariectomised before ova-implantation. Emphasis is placed on the time relationships between the start and interruption of normal pregnancy by ovariectomy. The establishment of continued pregnancy in ovariectomised mice with progesterone treatment then allowed a study of the survival rates and of the spacing of embryos within an altered maternal hormonal environment.

Accordingly the thesis presentation is divided into the three sections:

I. the ovarian hormonal requirements for ova-implantation;

II. the ovarian hormonal requirements for post-implantation survival;

III. the ovarian hormonal requirements for embryonic spacing within the uterus.

Section I includes a subsection (section I(a)) which deals with the time of ova-implantation in intact mice and a consideration of the health and mating behaviour of the experimental mice.

The purpose of this introduction is to provide a general framework on which each of the outlined aspects of reproduction can be more closely examined.

1. The hormonal Requirements for Ova-implantation

(a) Neuroendocrine considerations

The secretion of the main ovarian hormones, the oestrogens, oestradiol and oestrone, and the progestagen, progesterone (Pincus, 1965a) is under neuroendocrine control.

The ovary functions as an integral part of a complex neuroendocrine hierarchy, the hypothalamo-hypophyseal-gonadal axis (Everett, 1961). Evidence for the neural control of the ovary has been reviewed by Harris (1960), Everett (1961, 1964) and Guillemin (1964). Neural directions to the ovary are indirect and are initiated from the hypothalamus. Small polypeptides, or releasing factors, capable of stimulating the release of the hypophyseal gonadotropins, luteinizing hormone (LH) (Schally and Bowers, 1964) and of follicle stimulating hormone (FSH) (Igarashi, Nallar and McCann, 1964) have been isolated from beef and rat hypothalamic extracts, respectively. The releasing factors are probably synthesized in the hypothalamic neurones and released by neurosecretion into the hypophyseal vascular portal system to reach the adenohypophysis (B. Scharrer, 1967).

Of the two gonadotropins, FSH is considered to be primarily responsible for the phase of follicular growth (reviewed by Rowlands and Parkes, 1966) and an accelerated release of LH is probably the stimulus responsible for ovulation (reviewed by Everett, 1965; Ramirez and Sawyer, 1965).

Prolactin, the principal luteotropic hormone in rats and mice (Rothchild, 1966) is released from the adenohypophysis probably in response to a hypothalamic stimulus evoked during mating (Everett and Quinn, 1966). Prolactin is needed before the corpora lutea become competent to secrete sufficient progesterone to allow implantation to occur (reviewed by Eckstein and Zuckerman, 1956).

Although it is probable that both FSH and LH are required for the

secretion of oestrogen from the ovaries of the immature mouse (Eshkol and Lumenfeld, 1967) and immature rat (Lostroh and Johnson, 1966), LH alone was sufficient for the secretion of this hormone from the luteinized ovaries of the mature rat (Macdonald, Armstrong and Greep, 1966).

(b) Pré-implantation phenomena

After successful fertilization, the egg is subjected to a period of tubal passage during which cleavage occurs, and a period of intra-uterine existence during which development and growth of the blastocyst supervenes. While the rate of cleavage is independent of ovarian hormonal influence (Brinster, 1963) the rate of tubal passage is liable to ovarian hormonal changes. The effects of oestrogen and progesterone on tubal transport have been reviewed by Austin (1963), Adams (1965) and Pincus (1965b). Exogenous oestrogens can either speed, slow or stop ('tube-lock') egg transport (see Chang and Harper, 1966) while progesterone probably acts to slow transport during normal pregnancy. Egg transport is apparently normal in ovariectomised rats (Alden, 1942a) and in ovariectomised mice (Smithberg and Runner, 1956). The correct timing of the arrival of the eggs into the uterus is crucial if further development is to be successful. Premature arrival may lead to expulsion through the vagina, while delayed entry in the rat precluded the uterus from responding in a favourable way to the presence of the blastocyst (Psychoyos, 1966).

After entry into the uterus the eggs are spaced throughout the length of the uterine horns and in the mouse each comes to occupy an antimesometrial crypt (Snell, 1956). Ova spacing in the rabbit is about even and is thought to be an ordered event, in that the blastocysts and the uterus each play a part (Böving, 1956). There is some doubt as to how evenly the blastocysts are spaced in the mouse and uterine movements have been considered to play the major role in spacing the blastocysts (McLaren and Michie, 1959;

Wilson, 1963).

The intra-uterine requirements for ova-implantation are strict, both with regard to the development status of the ovum and the stage of hormonal preparation of the uterus. This is in marked contrast to the relative ease with which mouse ova will 'implant' in extra-uterine sites regardless of the endocrine condition of the host (Fawcett, Wislocki and Waldo, 1947; Kirby, 1965a).

In the nonlactating pregnant mouse and rat, the ova and the uterus develop in phase with one another. In these species progesterone acting after, though synergistically with oestrogen is the normal sequence of hormonal action during the period of free ovum existence (Courrier, 1950). The progesterone-dominated phase is characterised by endometrial proliferation, enhanced glandular secretion of 'uterine milk' and by reduced myometrial activity (see reviews by Mayer, 1960; and Reynolds, 1965). Rabbit blastocysts before implantation are rich in coenzymes and vitamins and are capable of a marked degree of metabolic selectivity (Lutwak-Mann, 1963). Ovariectomy immediately prior to implantation was associated with obvious disturbances in the metabolic behaviour and morphological changes in rabbit blastocysts and produced failure of implantation (Lutwak-Mann, Mays and Adams, 1962).

Implantation in several species (e.g. the mouse, rat and rabbit) is associated with the proliferation and differentiation of the uterine stromal connective tissue to form decidual tissue which ultimately provides an implantation chamber for each ovum (see reviews by Mossman, 1937; Amoroso, 1952; and Shelesnyak and Kraicer, 1963). Deciduomata, as decidual tissue localizations invoked by methods or agents other than implanting blastocysts are known, may be produced in the uteri of pregnant or pseudopregnant mice and rats by a variety of artificial stimuli during

a limited period of the pre-implantation, or progestational stage of pregnancy. It is during this limited period of uterine sensitivity that changes associated with implantation are initiated. Both in the rat and the mouse maximum uterine sensitivity to decidualizing stimuli follow the action of both progesterone and oestrogen (De Feo, 1963; Shelesnyak and Kraicer, 1963; and Finn, 1965, 1966a, respectively).

The function of decidual tissue is obscure and has been discussed by McLaren (1965). It may play a nutritive role as far as the blastocyst is concerned and a defensive role in protecting the uterus from trophoblastic invasion.

Oestrogenic and progestational hormones may not normally act independently of one another. Evidence for synergism and antagonism between the two hormonal groups has been discussed by Courrier (1950) and Hisaw and Hisaw (1961). Experiments have shown that the results of the interactions depend on both the absolute amounts and the ratio of oestrogen to progesterone present, the duration of the action, the target tissue and the species under consideration.

Pincus (1965c) has discussed the effectiveness of many steroidal hormones and nonsteroidal compounds in interrupting the progestational phase of early pregnancy. One of the most potent groups of antifertility agents are the natural oestrogens. Others active in this way are the so-called antioestrogens, pro-oestrogens, antiprogestagens and to a lesser extent progestagens. Their effectiveness would appear to result from their ability to alter the delicate balance and sequence of action of the endogenous ovarian hormones, either due to their own effects and/or to interference with the actions of endogenous oestrogens and progestagens. Among these substances can be found examples that inhibit proliferation of the endometrium, promote alterations in the motility of the

reproductive tract, inhibit deciduogenesis and alter the nature of tubal and uterine secretions.

(c) Methods of investigating the hormonal requirements for ova-implantation in rodents and the 'oestrogen surge' hypothesis

The methods used are broadly divisable into two main groups: studies concerned with normal pregnancy and its physiological variants; and studies involving the interruption of pregnancy and the dissociation of the blastocyst/uterus relationship.

Ovarian hormonal levels have been estimated during normal pregnancy by the largely subjective method of noting changes in the morphological characteristics of the reproductive tract and comparing these with the known changes in the response of these tissues to oestrogens and progesterone (reviewed by Deanesly, 1966). With the advent of more sensitive biochemical methods attempts have been made to estimate directly the plasma levels of progestagens in the pregnant rat (Fajer and Barraclough, 1967). Plasma oestrogen levels are low during pregnancy and Grota and Eik-Nes (1967) were unable to detect these with any accuracy during late pregnancy and early lactation in the rat.

Delayed implantation, during which the blastocysts remain free in the uterine lumen for an extended period, occurs naturally in some species and in lactating pregnant mice and rats. The length of the delay is roughly proportional to the number of suckling young and for lactating mice implantation is delayed about one day for each pup suckled (Enzmann, Saphir and Pincus, 1932; Turpeinen, 1943). Pregnant lactating mice have been used in attempts to define the hormonal needs for blastocyst survival and implantation (Bloch, 1958, 1959, 1965; Whitten, 1955, 1958).

Experimental studies on how ovarian hormones are involved in ova-implantation have concentrated on the uterus alone, to a lesser extent on

the egg alone and on the reciprocal relationships between the uterus and the blastocyst. Reversible interruption of implantation has been achieved by removal of or by altering the functioning of the endocrine glands that constitute the hypothalamo-hypophyseal-gonadal axis.

The artificial induction of delayed implantation in the rat has been responsible for much of the information that has accrued concerning the hormonal requirements for ova-implantation in this species. This work has been reviewed by Mayer (1963) and Psychoyos (1966). Implantation can be delayed by manipulation of the hypothalamo-hypophyseal-gonadal axis before the afternoon of day 3* of pregnancy so that the uterus remains in a 'neutral' progestational state. Maintenance of this progestagen-dominated phase can be effected by ovariectomy and the administration of exogenous progesterone (Cochrane and Meyer, 1957; Mayer 1959; Nutting and Meyer, 1963).

Hypophysectomy and autotransplantation of the hypophysis to a site remote from the hypothalamus (Everett, 1956) or the administration of tranquilizers (Psychoyos, 1963; Mayer, 1965) before day 3 of pregnancy delays implantation and both these procedures are compatible with the continued release of prolactin which in turn maintains ovarian progesterone secretion. The delayed blastocysts, free in the uterine lumen do not immediately die, in fact Cochrane and Meyer (1957) observed that blastocysts were maintained in a viable state for 45 days and Mayer and co-workers

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* The day on which evidence of mating is found (e.g. a vaginal plug, or sperm found in vagina) is defined as day 0. Results of other workers are adjusted to coincide with this usage.

found that rat blastocysts remained alive in the uterus for up to seven days in the absence of either ovarian or adrenal hormones (Mayer, 1959). Implantation can be brought about during the period of delay by the concurrent administration of progesterone and a small single dose of oestrogen.

From these and other studies there has arisen the concept of a neuroendocrine hierarchal control of ova-implantation (see reviews by Mayer 1965; Shelesnyak and Kraicer, 1963). The hypohysis, presumably under the influence of hypothalamic releasing factors, in particular LH - releasing factor releases LH which in turn acts on the ovary long enough to cause an ephemeral discharge of oestrogen late on day 3 of pregnancy. Oestrogen acting during the progestational phase of pregnancy and in particular an 'oestrogen surge' (proposed by Shelesnyak, 1959, 1960; see also Shelesnyak and Kraicer, 1963) is regarded as essential for implantation in the rat and causes the uterus to change from what Psychoyos (1966) calls the 'neutral' to the 'receptive' state and is also associated with changes in form of the blastocyst (Yasukawa and Meyer, 1966). Work by Macdonald, Armstrong and Greep (1967) and Hayashida and Young (1965), supported the involvement of the hypothalamus and in particular the release of LH in rats. The former authors found that rats that were hypophysectomised on day 1 of pregnancy and had their pituitaries autotransplanted were subsequently able to implant ova when given exogenous LH. While Hayashida and Young inhibited implantation by the daily injection of an antiserum to LH for 5 days, starting immediately after breeding. Studies by Zeilmaker (1963) and Psychoyos (1963) during which hypophysectomy and tranquilizers, respectively, were used to determine the time limits of pituitary involvement in implantation, showed that inactivation of the pituitary had to be completed before day 3 of pregnancy if implantation was to be delayed under conditions that allowed continued

progesterone secretion. The earliest time after which ovariectomy did not delay implantation, again in the advent of progesterone being available, was about 12 hours later (Mayer, 1963). This suggests that there is a definite time ordered sequence of action of the neuroendocrine factors controlling ova-implantation.

Comparatively, the mouse has been subjected to fewer investigations than the rat. However, recent work by Bindon and Lamond (1968) would suggest that as in the rat, pituitary involvement occurs during a definite period of early pregnancy. Hypophysectomy before this time together with progesterone administration prevented ova-implantation, while the same treatment later on in pregnancy was compatible with implantation. The effect of ovariectomy on implantation in the mouse is also less certain than in the rat and will be dealt with in detail elsewhere.

Ova transfer experiments with ovariectomised recipients have been used to establish the hormonal needs for subsequent implantation in the mouse (Humphrey 1967; Smith 1966) in the hamster (Orsini and Psychoyos, 1965) and in the rat (Dickmann, 1967).

Pseudopregnant animals have been used extensively to investigate the role of the uterus in ova-implantation, especially with regard to the onset, duration and hormonal control of uterine sensitivity to decidualizing stimuli in the rats (Shelesnyak and Kraicer, 1963; Shelesnyak, 1965; De Feo, 1963; Yochim and De Feo, 1963; Finn and Keen, 1962) and in the mouse (Finn and Hinchliffe, 1964, 1965; Finn, 1965, 1966a). The assumption generally made was that the maintenance of the corpora lutea of pseudopregnancy allowed the establishment of a hormonal environment simulating that of normal pregnancy. There are however, many ways in which to induce a state of pseudopregnancy and there is evidence to suggest that the actual endocrine conditions prevailing in pseudopregnant rats differ according to

the methods used to induce this state (Banik and Ketchel, 1965; Fajer and Barraclough, 1967). Further, the hormonal requirements for deciduomata formation may differ according to the decidualizing stimulus employed (Finn, 1965).

The development of ova in ectopic sites has allowed emphasis to be placed on the egg in the absence of the uterus (Fawcett, Wislocki and Waldo, 1947; Kirby, 1965a, b, 1966). Dickson (1966a) has studied changes in the form of the implanting blastocyst and compared these with changes occurring after ovariectomy in the mouse (Dickson, 1966b, c). Also Yasukawa and Meyer (1966) have noted oestrogen-dependent changes in the shape of rat blastocysts prior to implantation.

2. Embryonic Survival

Variations in the reproductive performance of the female mouse can result from changes in the number of eggs ovulated, the fertilization rate, the implantation rate and in the embryonic and foetal survival rates. This present study is concerned with the implantation rate and post-implantation survival until day $12\frac{1}{2}$ of pregnancy when the endocrine balance has been upset by ovariectomy before implantation and attempts made to correct this state by the administration of exogenous hormones. Emphasis is placed on the relative rates of survival between intact and ovariectomised pregnant mice rather than on the magnitude of the prenatal losses. An approximate guide as to the efficiency of reproduction can be derived from a comparison between the numbers of corpora lutea and the number of implanted embryos. Further, of the many casual factors able to lower the efficiency of reproduction only those referable to a defective maternal endocrine balance will be considered.

(a) Pre-implantation Survival

Social influences, mediated by olfactory sensitive pheromones (literally

'carriers of excitation') influence the oestrous cycle and the luteotropic process in the mouse (see review by Bruce and Parkes, 1965). The pheromones are probably produced under the influence of testosterone and secreted in the urine of male mice (Dominic, 1965; Bruce, 1965). The exposure of recently mated female mice to 'strange males' especially those from another strain, leads to failure of both pregnancy and pseudopregnancy (the 'Bruce Effect'). It is likely that the pheromones inhibit the endogenous release of prolactin because the administration of this luteotropin allows the female to maintain her pregnancy (Bruce and Parkes, 1960).

Progesterone administration was required before implantation occurred in intact prepubertal mice stimulated to ovulate and mate after gonadotropin treatment (Smithberg and Runner, 1956). It has been observed that daily handling of pregnant mice reduced the percentage of mice that remained pregnant (Runner, 1959) and that inbred mice that showed vaginal plugs often did not show implantation sites (Runner, 1960). In each case, failure of the animals' luteotropic processes was thought to be responsible for the pregnancy failures. Progesterone administration prevented the embryocidal effect of handling the mice and actually allowed a higher percentage of these animals to maintain their pregnancies than of the nonhandled controls. Pregnancies were recorded at 18 days post coitum or by the birth of living young so some losses may have been post-as well as pre-implantational.

The stresses of high temperature and hypoxia inhibit implantation in the intact but not in the adrenalactomised rat, suggesting that implantation may have been prevented by adrenal hormones (Fernandez-Cano, 1959). The combined stresses of suckling two young and burning on the hindleg of nursing rats on the day 3 of pregnancy caused a delay in implantation, whereas acting individually, neither stress delayed implantation (Canivenc and Mayer, 1955a, 1955c). McClure (1963) found that starvation of mice

and rats at or about the time of implantation prevented pregnancy.

(b) Post-implantation survival

Amoroso (1955) and Deanesly (1966) have reviewed literature concerned with the role of the endocrine glands during pregnancy. There is a changing relationship between the components of the hypothalamo-hypophyseal-gonadal axis as gestation progresses, due largely to the ability of the placenta to secrete hormones. Available evidence suggests that perhaps the uterus should be considered as an integral part of this axis. When acting via a local (Bland and Donovan, 1965; Melampy, 1966) or by way of a systemic influence (Nalbandov, 1966; Greep, 1966), the uterus is able to influence the life span of the corpora lutea and thus the availibility of progesterone. In both the guinea pig (Bland and Donovan, 1965) and the mouse (Kirby, 1965b), it has been argued that as a result of the interaction between the trophoblast and the decidual tissue, luteal function is stimulated. In both these species, ectopic pregnancies do not influence the oestrous cycle and Kirby (1965b) has shown that mice are able to carry extra- and intrauterine pregnancies concurrently. Luteotrophic substances have been demonstrated from both mouse and rat placentae (Amoroso, 1955; Deanesly, 1966) and are probably responsible for the maintenance of pregnancy in the mouse after hypophysectomy in the latter half of gestation (Gardner and Allen, 1942). Fajer and Barraclough (1967) measured an increase in the amount of ovarian progestagens secreted on day 13 of pregnancy in the rat and suggested that it may be due to the activity of placental luteotropin. There is indirect evidence that the rat and mouse placentae are able to secrete oestrogens and small amounts of progestagens during pregnancy (see Deanesly, 1966).

Removal of the ovaries before or after implantation usually interrupts pregnancy in the rat and the mouse. Substitutional treatment with

progesterone has been effective in maintaining viable foetuses until late pregnancy in the mouse (Hall and Newton, 1947; Smithberg and Runner, 1956; Rubenstein and Forbes, 1963; Poulson, Sullivan and Robson, 1965), rat (Lerner, Brennan, Yiacus, De Phillipo and Borkman, 1962), rabbit (Pickworth, 1963) and hamster (Harper, Prostkoff and Reeve, 1966). The proportion of implantation sites that are alive at any stage of pregnancy provide a more sensitive indicator of the effectiveness of the hormonal therapy than is provided by the number of mice with viable embryos. Oestrogen synergises with progesterone to maintain pregnancy in ovariectomised animals. (Courrier, 1950; Lerner et al., 1962, Harper et al., 1966).

There is some evidence that progesterone and oestrogen treatments do not always provide adequate support to allow normal foetal development in ovariectomised rats (Carpent, 1962). The time of ovariectomy, the doses of progesterone and oestrogen used and the strain of rats were found by Carpent to be important (see Chambon and Le Vève, 1966).

(c) Embryopathic effects associated with oestrogens and progestagens

Both gross and visceral foetal abnormalities have resulted from the artificial creation of endocrine imbalances during gestation.

Carpent (1962) describes gross malformations probably caused by the compression of the embryos or foetuses due to 'uterine hypertonicity', following ovariectomy and administration of progesterone and oestrogen to pregnant rats. In earlier work, Selye, Collip and Thomson (1935) and Zeiner (1943) believed that the uteri of ovariectomised rats contracted and that as a result the foetuses were compressed. Poulson, Robson and Sullivan (1965) noted that a minority of the foetuses carried by mice that were ovariectomised during embryogenesis and maintained on low levels of progesterone showed gross abnormalities.

Visceral malformations that were probably not secondary to uterine

changes have been found in rat foetuses by Carpent and Desclin (1967). Cardiovascular and ocular defects were seen in the few surviving foetuses from rats that were hypophysectomised and given a grafted pituitary gland early in pregnancy and then maintained with deficient hormonal substitution treatment.

Exogenous oestrogens are able to terminate pregnancy and induce modifications of the genitalia of both male and female foetuses in mice and rats (reviewed by Deanesly, 1966). The effective dose rate of oestrone needed to terminate pregnancy in the rat rises considerably after implantation (Edgen and Shipley, 1961). High levels of progesterone administered to pregnant mice were able to increase the prenatal mortality rate (Fowler and Edwards, 1960) and when given either subcutaneously or by intra-amniotic injection can cause foetal death (Petrelli and Forbes, 1964).

(d) Local uterine influences

The position, the number and the distances apart of foetuses in the uterine horns of the pregnant mice are reported to influence their growth and/or mortality rates. The body weight of foetuses closest to the oviducts was found to be significantly lighter than that of its neighbour (Hashima 1956; McLaren and Michie, 1959). Hollander and Strong (1950), investigated the mortality rate in relation to the number of foetuses per cornu and found that low and high numbers were associated with a higher mortality rate than were intermediate numbers. Placental fusion that followed close embryonic spacing was associated with the reduced growth rates of the surviving foetuses whose placentae were joined (McLaren and Michie, 1959).