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The Physiology of the Ovary: Maturation of Ovarian Granulosa Cells and a Novel Role for Antioxidants in the Corpus Luteum

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During folliculogenesis the granulosa cells divide whilst in contact with each other, and so exhibit some of the characteristics of stem cells. *In vitro* we have shown that bovine granulosa cells from 3–7 mm follicles, like stem cells, divide without the need for a substratum, and produce colonies of cells. Growth factors, bFGF and IGF's, stimulate their division. These cells secrete and assemble a basal lamina, suggesting that the follicular basal lamina is produced by the granulosa cells. They have the morphological characteristics of follicular granulosa cells. Thus this system is ideal for studying the functions of immature granulosa cells because the cells do not spontaneously differentiate or luteinize into luteal cells, as occurs in culture on a substratum. On differentiation into luteal cells *in vivo* the cells express the steroidogenic enzymes for progesterone production and accumulate β-carotene. During culture of bovine luteal cells we observed that a proportion of the steroidogenic enzyme cholesterol side-chain cleavage cytochrome *P*450 enzyme became chemically cross-linked to its electron donor, adrenodoxin. *P*450 enzymes produce oxygen free radicals and oxygen free radicals can cause cross-linking between proteins in close proximity. Cell protect against this damage by the use of antioxidant vitamins. Repleting the cultured luteal cells with β-carotene reduced the amount of cross-linking. We conclude that the high levels of β-carotene in corpora lutea are to protect against damage due to oxygen free radicals generated in the course of progesterone synthesis.

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INTRODUCTION

Being asked to present on such a broad topic as the "physiology of the ovary" gives one the opportunity to discuss results on more than one topic. It is becoming well understood now that the study of the endocrinology of the ovary is more about the growth and maturation of the ovarian endocrine organs, follicles and corpora lutea, within the ovary. This is because the changes in the hormone secretion by the ovary on a day to day basis is unique amongst endocrine organs. The changes are due to the formation or regression of the endocrine organs rather than simply an alteration in the secretion of hormones from preexisting endocrine cells. It is for this reason that we and others are interested in the development of follicles and corpora lutea. Two aspects of this will be discussed, firstly the growth of bovine follicles and secondly the role of antioxidants in bovine corpora lutea.

MATURATION OF GRANULOSA CELLS

In mammals the ovary acts as a reservoir of eggs and is the source of hormones which control the phases of the reproductive cycle, ovulation and pregnancy. The number of eggs or oocytes within the ovary is determined during fetal life as oogonia undergo a series of divisions to produce oocytes arrested in meiosis. At this stage they are embedded in the ovary, surrounded by flattened epithelial-like cells in primordial follicles. Each day during adult life a few of these arrested oocytes (4–8 in sheep) become active [1]. They enlarge in size and the single layer of surrounding flattened epithelial-like cells become cuboidal in shape and

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commence dividing. These cells are the granulosa cells of the growing follicle, and these cells divide over a period of months [2] as the follicle matures. An obvious but not well appreciated fact is that these granulosa cells divide whilst within ovarian stroma, and thus in contact with themselves and stromal cells. This is an important feature of these cells for most other cell types in adults, other than tumour cells or stem cells, do not divide when in contact with other cells. We postulated that the granulosa cells could have some of the characteristics of stem cells or tumour cells whilst dividing during development of the follicle. We therefore investigated the division and differentiation of granulosa cells under anchorage-independent conditions [3, 4]. Without anchorage or attachment to a substratum most cells, such as fibroblasts, cannot divide, where as stem cells and tumour cells can. The results were surprising.

Briefly granulosa cells were gently scraped from small and medium sized bovine follicles (3–7 mm diameter). Using a modification of the method of Bertoncello *et al.* [5] cells were then aliquoted onto a prepoured 0.5% agar base in MEM with either 10% or 20% fetal calf serum and antibiotics. The cells were overlayed with 1% methylcellulose in the same medium as the agar base. The cells were then cultured under these conditions (control) or with the addition of basic fibroblast-like growth factor (bFGF), insulin-like growth factor-I (IGF-I), or dibutyryl cAMP. The cells were cultured (up to 14 days) at 37 °C in a humidified atmosphere of 7% O_2/10% CO_2/83% N_2. At the end of each culture period the cells were either pulse radiolabelled with [^{3}H]thymidine [4], for estimation of cell division, or harvested for estimation of DNA content [3], for Western immunoblotting, for immunohistochemistry or for light and electron microscopy [3]. The supernatants were also harvested for hormone assays [4].

Under either control conditions or in the presence of growth factors the cells divided and grew as colonies [3, 4]. Thus the cells exhibited some of the properties of stem cells or tumour cells which *in vivo* would be observed as a lack of contact inhibition of cell division. Only a proportion of granulosa cells from 3–7 mm follicles have these properties though, because far fewer cells divided under anchorage-independent conditions than did in monolayer, as measured by [^{3}H]thymidine incorporation into DNA. Both bFGF (Fig. 1) and IGF-I enhanced the rate of cell division of the granulosa cells [3, 4], with dibutyryl cAMP treatment cell inhibiting division within days [4]. These observations suggest follicular growth factors can stimulate contact uninhibited growth of granulosa cells *in vivo* and that the cAMP, the second messenger of luteinizing hormone, can inhibit it.

Under control conditions and in the presence of growth factors the granulosa cells had the features of follicular granulosa cells (Fig. 2). They were small with a relatively unfolded cell membrane, with a low nuclear/cytoplasmic ratio, with few mitochondria, and with a small amount of endoplasmic reticulum that was mostly rough. Gap junctions were present between the cells. This discovery represents a major advance in this area for the other commonly used culture system of monolayer, induces or allows the granulosa cells to spontaneously differentiate or luteinize into luteal cells [6], thus confounding the study of their follicular functions. How or why this spontaneous differentiation occurs is not known at this stage.

No oestradiol production could be detected under control conditions, even in the presence of follicle-stimulating hormone or androstenedione as substrate [3]. Since granulosa cells *in vivo* only produce oestradiol when the follicles are mature we assume that the cells that divided under anchorage-independent conditions were more immature. The production of oestradiol has become the standard expectation of granulosa cells cultured as monolayers and in particular during the first day in culture [7]. However, *in vivo* oestradiol is only produced by the granulosa cells of large antral follicles [8]. Follicles of this size remain in the bovine ovary for at most only a week or two [9] before undergoing atresia or ovulating; both processes leading to a reduction in oestradiol production [8, 10]. Thus granulosa cells probably spend only 5% of their life span producing oestradiol. Most of their life time [1, 2, 11] is spent as a more immature dividing cell. It is important then that we be able to study this phase of granulosa cell function, and anchorage-independent conditions will be a useful tool for this.

In the control and growth factor-stimulated colonies large amounts of an extracellular matrix were observed

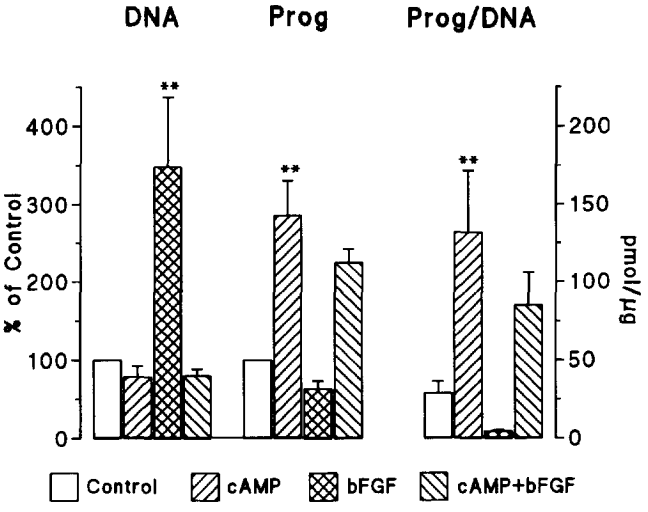

Fig. 1. The mean ± SEM levels of DNA and progesterone (expressed as either a percentage of control levels or per µg DNA, where control = 31 ± 7.2 µg/dish). Granulosa cells were cultured for 14 days either under control conditions, or with the addition of 1 mM dbcAMP, 50 ng bFGF/ml or both. $^*P < 0.05$, $^{**}P < 0.01$ significantly different from controls, paired analyses ($n = 6$ experiments, 3 replicates per experiment). Reprinted with permission from Lavranos *et al.* [3].

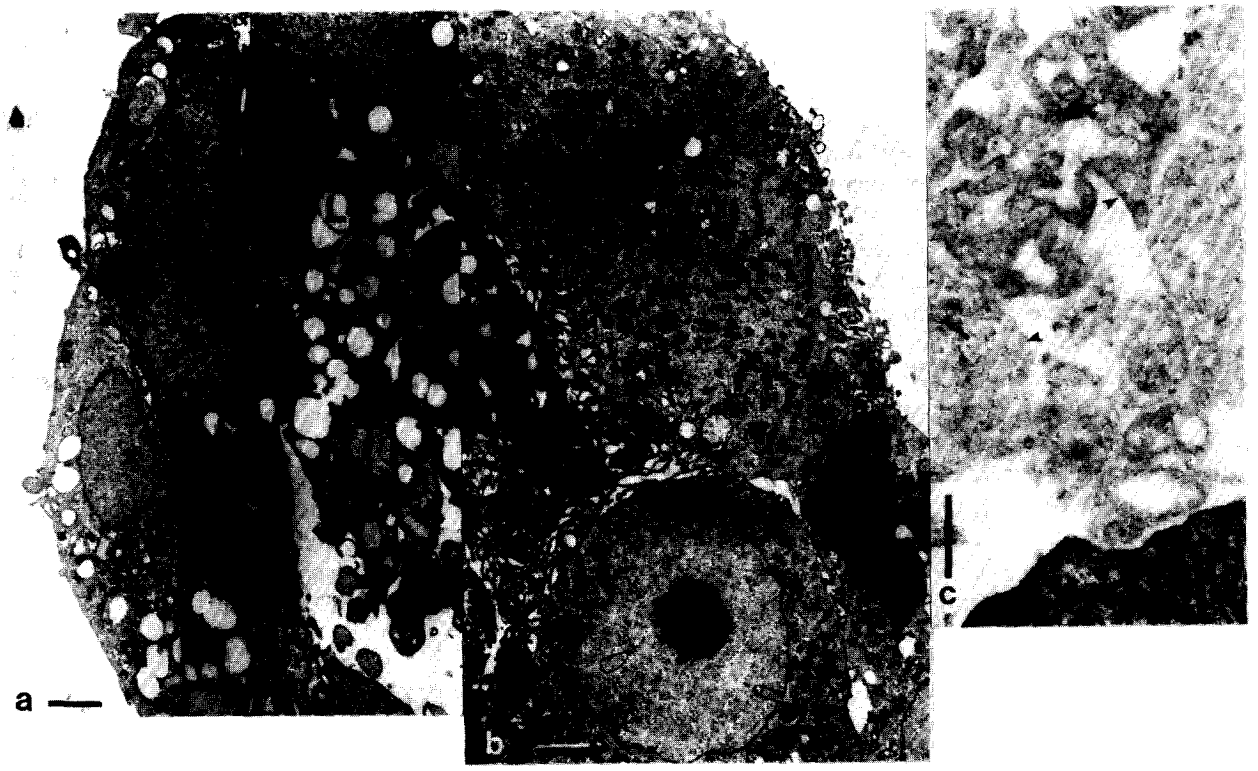

Fig. 2. Electron micrographs of cells incubated for 7 days under either control conditions (a) or in the presence of 1 mM dbcAMP (b) or of the extracellular matrix produced by cells incubated with 50 ng bFGF/ml (c). L indicates lipid droplet and arrows indicate dilated rough endoplasmic reticulum in (a) and extracellular matrix in (c). Bar sizes in a, b and c are respectively 2 μm, 2 μm and 0.5 μm. Reprinted with permission from Lavranos *et al.* [3].

[3]. At the electron microscope level it had the features of assembled basal lamina (Fig. 2). Components of basal lamina, fibronectin and collagen type IV, were immunolocalized to the colonies. Fibronectin was of a higher molecular weight than the circulating form found in bovine plasma, and bFGF stimulated fibronectin production. *In vivo* a basal lamina surrounds the membrana granulosa, separating it from the surrounding stroma and theca (see Fig. 1 of [12]). This follicular basal lamina is presumably synthesized by the adjacent granulosa cells, for it is in close apposition to these cells. The basal lamina produced by the granulosa cells under anchorage-independent conditions does not neatly align parallel to the cell surface but rather gives the appearance of being oversecreted and accumulating within the colony of cells. It may also accumulate outside of the colony and be merely lost during the harvesting of the colonies. However, the over secretion of the basal lamina towards the centre of the colony is similar to that seen by the mouse embryonal carcinoma cell line, PCC4-F, when cultured in solution without anchorage [13]. The structural features of the extracellular matrix observed are not dissimilar to those observed in the basal lamina of a renal tubular epithelium of diabetic patients [14]; patients who are known to have extensive basal lamina of diverse forms [14]. Also consistent with the oversecretion of basal lamina by the granulosa cells, was their dilated rough endoplasmic reticulum containing a flocculent-like material. The EHS tumour cells that secrete large amounts of laminin, an integral component of basal lamina, also exhibit these same cellular features [15]. Anchorage-independent culture of granulosa cells will thus allow us to study the production of the follicular basal lamina, without the confounding production of basal lamina by the endothelial cells of the thecal capillaries.

Under dibutyryl cAMP treatment the cells hypertrophied into cells with the features of large luteal cells (Fig. 2). Other features indicative of differentiation into large luteal cells included an enhancement of progesterone production above control levels [3], and in the levels of cholesterol side-chain cleavage cytochrome *P*450 and 3β-hydroxysteroid dehydrogenase as occurs *in vivo* following ovulation [16,17]. Basal lamina and fibronectin were not detected in these cultures.

In conclusion we have shown that granulosa cells divide and maintain their follicular functions under anchorage-independent culture conditions and that dibutyryl cAMP can stimulate their differentiation. This system will be useful for studying the control of granulosa cell division and differentiation and for purifying and identifying the roles of the extracellular matrix produced by these immature granulosa cells *in vitro*.

A NOVEL ROLE FOR ANTIOXIDANTS IN THE CORPUS LUTEUM

Pregnancy is supported by the secretion of progesterone from the ovarian corpus luteum [18, 19]. For reasons not well understood, the corpus luteum, which has a very high capacity to synthesize and secrete steroid hormones, is rich in antioxidants, particularly β-carotene [20, 21]. The levels are so high that the β-carotene gives the corpus luteum its characteristic bright yellow colour and hence its translated name of "yellow body". Not all species have a bright yellow corpus luteum though, but these corpora lutea of other species have high levels of other antioxidant vitamins. For example the corpus luteum of the rat is exceedingly rich in ascorbic acid [22]. As well, another very steroidogenic organ, the adrenal cortex, is also rich in antioxidant vitamins [22]. Current studies of antioxidant vitamins in steroidogenic tissues are focusing on a role for them in preventing lipid peroxidation [see 23]. This is not unreasonable as steroidogenic organs either have a high capacity for lipid metabolism or uptake or both in order to derive cholesterol for steroidogenesis.

However, there are many other things in common to steroidogenic tissues such as the first committed and rate-limiting step in steroidogenesis. This step is the conversion of cholesterol into pregnenolone, catalysed by the mitochondrial cholesterol side-chain cleavage cytochrome *P*450 enzyme (*P*450scc, *P*450XIA1) [18, 19]. Electrons for this process are derived from NADPH via an electron transport chain, composed of adrenodoxin reductase and adrenodoxin (ferredoxin) [18, 19]. *P*450 enzymes [24], including *P*450scc [25], are well known for producing oxygen free radicals and thus it would not be unreasonable that the high levels of antioxidants are present in steroidogenic tissues to protect against such oxygen free radicals [22, 26, 27].

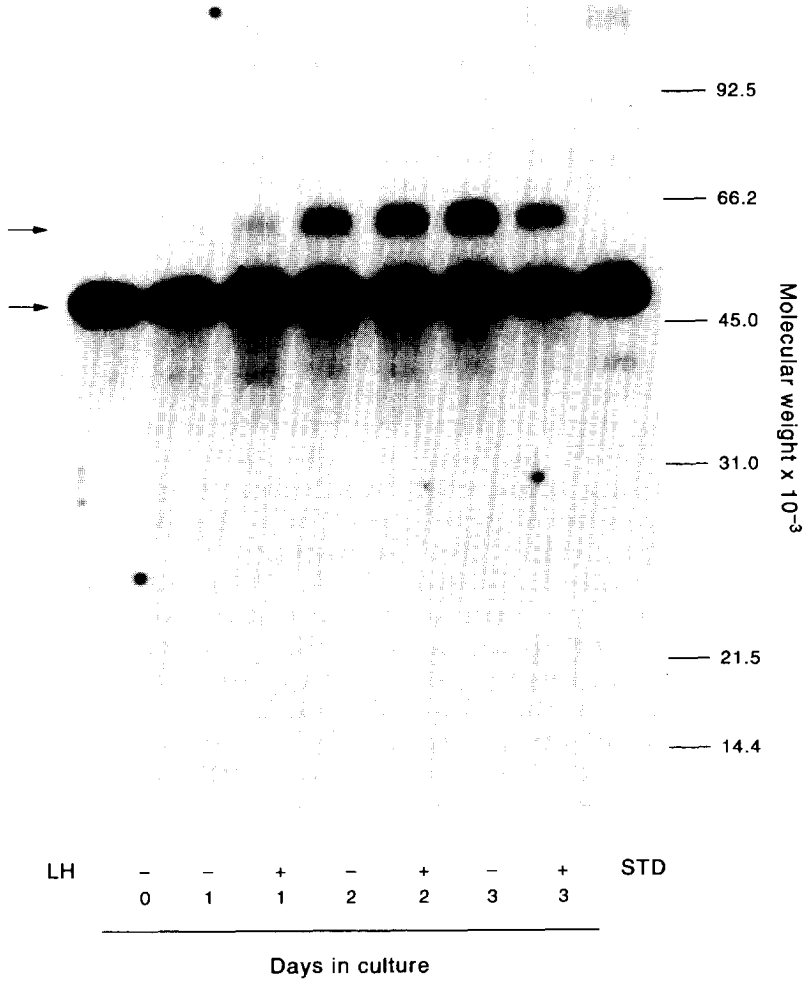

Fig. 3. The cross-linking of *P*450scc and adrenodoxin during culture. Autoradiograms of a Western immunoblot developed using anti-*P*450scc, of purified standards (STD) and proteins (50μg per lane) from luteal cells cultured with (+) or without (−) bovine luteinizing hormone (LH) for either 1, 2, or 3 days (24 h, 48 h, 72 h exactly). The lower arrow indicates the position of the purified standard and of the native protein. The upper arrow indicates the position of the *P*450scc-adrenodoxin cross-linked protein referred to in the text. The position of the molecular weight markers is shown on the right. Reprinted with permission from Young *et al.* [28].

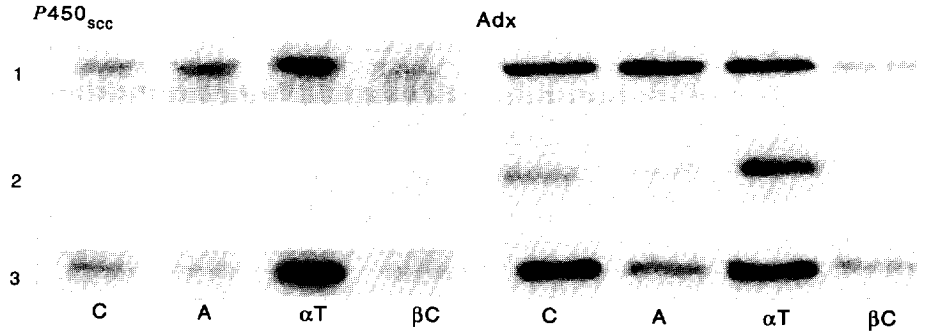

Fig. 4. The effects of antioxidants on *P*450scc–adrenodoxin (Adx) cross-linking. Autoradiograms of Western immunoblots were developed using either anti-*P*450scc or anti-Adx sera of bovine luteal cells cultured under control conditions or in the presence of ascorbic acid (A, 50 μM), α-tocepherol (αT; 25 μM) or β-carotene (βC; 5 μM; vehicle alone had no effect, $n = 3$) showing only the *P*450sccAdx cross-linked proteins from three separate experiments (1, 2, 3). Reprinted with permission from Young *et al.* [28].

In a study of *P*450scc (49 kDa) and its *P*450-specific electron donor, adrenodoxin (approx. 14 kDa) in bovine luteal cells in culture, both antisera to *P*450scc (Fig. 3) and to adrenodoxin, apart from recognizing their respective proteins, recognized a protein of higher molecular weight (63 kDa) in their respective immunoblots [28]. We concluded that these proteins identified in immunoblots for *P*450scc and for adrenodoxin were in fact the same protein composed of *P*450scc and adrenodoxin covalently non-disulfide cross-linked in a one to one ratio. This is the same ratio as occurs when adrenodoxin binds to *P*450scc during electron transfer [29]. Aminoglutethamide, a specific inhibitor of *P*450scc activity [30], inhibited the degree of the cross-linking [28], further supporting our conclusion. Oxygen free radicals, particularly the hydroxyl radical ($^{\cdot}$OH) [31, 32] can cause covalent non-disulfide cross-linking between proteins [31, 33, 34] by oxidation of a tyrosine residue of one protein that in turn forms a covalent bond with that of another protein in close proximity [31, 33, 34] we hypothesized that the cross-linking between *P*450scc and adrenodoxin could have been caused by oxygen free radical damage for two reasons: firstly because adrenodoxin comes into close proximity and binds to *P*450scc in order to transfer an electron [35] and secondly, *P*450 reactions [24] including the cholesterol side-chain cleavage reaction [25], produce oxygen radicals.

Cells protect against damage by oxygen free radicals by use of enzymes (superoxide dismutase, catalyase and glutathione peroxidase) or by antioxidants or free radical scavengers (vitamins A, C or E or the vitamin A precursor, β-carotene) [22–24, 26, 27], which act as a sink for the electrons and energy of oxygen free radicals. To test the hypothesis that these exceedingly high levels are present to prevent *P*450scc–Adx cross-linking, luteal cells were cultured and the effects of added antioxidants examined. Under control conditions the levels of vitamin E and β-carotene declined by about 50% during the first day of cell culture and remained low. Cross-linking occurred. When β-carotene was added at a concentration similar to that found in bovine serum [20, 21] the amount of cross-linking was substantially reduced (Fig. 4). Adding vitamin C or E had no consistent effect (Fig. 4). Thus we concluded [28] that the antioxidant β-carotene is found in very high concentrations in the corpus luteum to prevent this cross-linking between *P*450scc and adrenodoxin. This type of cross-linking would of course render the *P*450scc enzyme inactive, for in the cell adrenodoxin normally dissociates from the *P*450 after electron transfer and reassociates with the other member of the electron transport chain adrenodoxin reductase, in order to receive another electron [36].

Thus the logical conclusion, but one that has still to be tested *in vivo*, is that the high levels of β-carotene in the bovine corpus luteum are necessary for progesterone synthesis and thus fertility.

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