UNDERLYING PHYSIOLOGICAL MECHANISMS CONTROLLING THE REPRODUCTIVE AXIS OF RABBIT DOES

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ABSTRACT

An increasing body of evidence unequivocally points to a strict inter-link between nutrition and reproduction. The paradigmatic case of leptin is examined to uncover nutritional-related physiological, cellular and molecular mechanisms. The presence of leptin receptors (Ob-R) in the ovary, oviduct, hypothalamus and anterior pituitary suggests that leptin is involved in a large array of regulatory actions at different levels of the hypothalamus-pituitary-ovary (HPO) axis of the rabbit. In luteal tissue, leptin increases prostaglandin (PG) F2α synthesis but inhibits progesterone release using, respectively, the JAK/STAT and MAPK pathways. In the oviducts, leptin inhibits PGF2α, but stimulates PGE2 synthesis. A nutritional challenge, evoked by 48-h fasting before AI, depresses all the reproductive parameters, reduces estradiol-17β pulse frequency and amplitude, lowers LH peak surge following GnRH injection, down-regulates the expression of oestrogen receptors (ER) in anterior pituitary, and causes a fall in plasma leptin concentrations. Although leptin secretion seems to be regulated by caloric intake rather than by adipose mass, taken together these findings support that leptin may act as a metabolic signal to switch on or off reproductive activity.
Stress-related mechanisms are briefly discussed from the perspective of the potential benefit derived by modulating the neuroendocrine as well as the immune system during early development.
Regarding the luteal function, several different luteolytic and luteotrophic forces at work as well as many levels at which these opposing and balancing influences may exert, via paracrine and/or autocrine mechanisms, their action in the corpus luteum (CL). The emerging role of the nitric oxide (NO)/NO synthase (NOS) system in the regulation of rabbit CL function is presented. Both endothelial and inducible isoforms of NOS are expressed at gene and protein levels in the CL, eNOS being regulated during pseudopregnancy. PGE2 exerts its luteotrophic effect through NOS down-regulation, while the PGF2α luteolytic effect is mediated by NOS up-regulation both *in vitro* and *in vivo*. During luteolysis, luteal cytokines may be involved in the up-regulation of NOS activity, while downstream NO may inhibit steroidogenesis and induce, after removal of the protective action of progesterone, the expression of p53 gene, a transcriptional regulator of apoptotic genes. First evidence for the action of endothelin–1 (ET-1), a potent vasoconstrictor synthesized by vascular endothelium, in promoting the luteolytic cascade mechanism in rabbits is enlightened.

Key words: reproductive axis, leptin, luteolysis, stress, rabbit.

INTRODUCTION

Reproduction represents a large area of interest for the physiology which, traditionally, is involved in the study of living organism, or any part of it, down to the basic underlying mechanisms that control their function. In the female, it encompasses several processes that span from follicular development to ovulation, from fertilisation to embryogenesis, from implantation to parturition and lactation, all of which are under hormonal control.
Several hormones produced by the hypothalamus, pituitary, and ovary (the HPO axis) come into play in a precisely and co-ordinate fashion to control ovulation and sexual behaviour via a series of complex feedback mechanisms (RAMIREZ and LIN, 1994). Rabbits do not have a well defined oestrous cycle, although waves of follicles continuously develop and regress at 7-10 days intervals under the tonic action of FSH (FLEMING *et al*., 1984). Ovulation depends upon the LH surge and occurs 9-10 hours after it. In rabbits, ovulation is a neuroendocrine reflex triggered by copulation. Mating activates diverse sensory areas, whose evoked signals converge, via neural pathways, over an integration centre of the hypothalamus (KAYNARD *et al*., 1990; LIN and RAMIREZ, 1991). The signals follow different neural routes through the brain stem and project also in several brain regions, including telencephalic structures and hyppocampus. In spontaneous ovulator species, many of the pre-motor neurones are oestrogen-sensitive and are therefore critical for mediating the effects of blood oestrogen and progesterone on the gonadotropin releasing hormone (GnRH) neuronal network within the cyclic LH surge-generator subsystem. In rabbits, the reflex LH surge-generator neuronal apparatus is usually unresponsive to the positive feedback by oestrogen (DUFY-BARBE *et al*., 1978). However, the GnRH neuro-secretory system is very responsive to a variety of neuromodulator signals relating information about internal and environmental stimuli which are relayed by a large array of olfactory, visual and auditory receptors and other central sensors involving both adrenergic and cholinergic components (SAWYER *et al*., 1974). The neuronal limb of the ovulatory reflex can be bypassed by the exogenous administration of GnRH that acts on the anterior pituitary to release LH within few minutes following binding to GnRH receptors, or by injection of hCG that directly targets mature follicles within the ovary. This basic knowledge made possible the introduction of the artificial insemination (AI) technique in rabbits which is nowadays largely diffused.
Several factors such as management, nutrition, and environment may influence the basic hormonal loops governing the HPO axis by way of mechanisms and interactions not completely understood. Disruption of the gonadal axis may happens at any level involving the hypothalamic centers responsible for GnRH pulse release (PAU *et al*., 1986) and control of sexual behavior (WADE *et al*., 1986), the pituitary cells responsible of gonadotropin secretions, and the ovarian cells, both somatic, responsible for hormone production, and gametes. Thus it is not surprising that poor fertility has many different causes, many different underlying factors feeding into it, which may turn out exceedingly complicated to disclose. Whereas the common reproductive indexes (fertility rate, pregnancy rate, prolificacy), may give valuable information about the consistency of a fertility problem, little doubt that more sophisticated techniques are needed for a better understanding of the underlying causes which may affect the HPO axis. We should be aware, however, that striking specie-specific differences do exist in many aspects of the reproductive system and, thus, inferences from results obtained in other species should be taken with caution without previous tests in the rabbit. The pituitary function can be

conveniently evaluated by examining the changes in circulating gonadotropins prior and after GnRH administration or coitus. Similarly, the ovarian activity may be appropriately assessed by the assays of steroid hormones. Nevertheless, *in vitro* experiments are often required for the study of physiological mechanisms. Moreover, the recent introduction of sensitive biochemical techniques for the study of gene and protein expressions related to hormones and their receptors has greatly widened the spectrum of research on reproduction down to the cellular and molecular level and opened new perspectives in this field.

So, through a stricter integration between biology and applied animal science, we are now gaining new insights needed for more effective treatments and better tailored strategies for improving reproduction in rabbits even if several details are still unresolved. However, in order to restrict the scope of this presentation, emphasis will be given to review the progress done in the recent past for uncovering those nutritional-related physiological, cellular and molecular mechanisms which are supposed to modulate the hypothalamus-pituitary-ovary axis via leptin's action as a paradigmatic example. Two other sections on stress-related mechanisms and potential luteotrophic and luteolytic factors which may control the life span of corpora lutea complete this review.

Nutritional-related mechanisms

That body weight and nutritional factors may affect reproduction, an extremely high anabolic process, is well known since long time and fasting-induced anestrous has been reported in several animal species (DISKIN *et al*., 2003). Thus, strong mechanisms are at work to inhibit reproductive activity when food availability is low or body energy reserves are inadequate to fulfil energy expenditure (BRONSON and MANNING, 1991; PRUNIER and QUESNEL, 2000; ARMSTRONG *et al*., 2003). This latter condition is often encountered in young does whose reproductive potential, already compromised by the endocrine antagonism of prolactin (THEAU-CLÉMENT and ROUSTAN, 1992), is worsened by the negative energy balance occurring when voluntary feed intake does not cope their requirements for lactation and concurrent pregnancy (PARTRIDGE *et al*., 1986; XICCATO, 1996; FORTUN-LAMOTHE and PRUNIER, 1999; PASCUAL *et al*., 2000; XICCATO *et al*., 2004). The effect of long-term caloric deficiency during development on the regulatory mechanisms of metabolism has been investigated only recently in rabbits by ROMMERS *et al*. (2002; 2004), but how these metabolic and hormonal signals affect the HPO axis and influence subsequent reproductive performance needs further research.

Although the causal link between caloric intake and fertility is well known, precisely how nutritive resources act on reproduction remains unclear. Several hormones work together in linking growth, metabolism, energy homeostasis, and reproduction (HORNICK *et al*., 2000). Between these, leptin, a 16.5 kDa cytokine encoded by the *obese* gene (*ob*), is thought to be an important component not only in the long-term regulation of body weight and body fat mass content, but also in the control of reproductive function (CUNNINGHAM *et al*., 1999; CLARK and HENRY, 1999; WILLIAMS *et al*., 2002; BRANN *et al*., 2002; STEINER *et al*., 2003). Leptin is primarily secreted by adipocytes (ZHANG *et al.,* 1994), but is also synthesised by many tissues of the female reproductive system such

as ovary, endometrium, placenta, and mammary glands (GONZALEZ *et al*., 2000; RYAN *et al*., 2002).

The action of leptin is transduced through the binding with a specific receptor, the Ob-R, which is a single membrane-spanning receptor and a member of the cytokine receptor superfamily (BJØRBÆK *et al*., 1997). The expression of the Ob-R gene was identified by reverse transcriptase polymerise-chain reaction and immunohistochemical technique in various tissues of the HPO axis (SPICER and FRANCISCO, 1997; KARLSSON *et al*., 1997; FINN *et al*., 1998; MORASH *et al*.,1999; JIN *et al*., 2000; LIN *et al*., 2000; RUIZ-CORTES *et al*., 2000; RYAN *et al*., 2002).

Cellular and molecular mechanisms of leptin

Although considerable progress has been achieved in understanding the reproductive actions of leptin over the past few years, only recently, however, the reproductive role of leptin has been explored in rabbits with some details by *in vitro* and *in vivo* studies (DALL'AGLIO *et al*., 2003; ZERANI *et al*., 2004; BRECCHIA *et al*., 2004).

The presence of leptin receptors in different ovarian structures of rabbits, including corpora lutea (CL) and follicles at different stages of development, suggests that leptin may have a role in the steroidogenesis of pre- and post-ovulatory follicles (ZERANI *et al*., 2004). Leptin was found to decrease progesterone basal release by rabbit CL through the JAK/STAT pathway in accordance with previous studies carried out on theca, granulosa and luteinised granulosa cells of rodent, bovine and primate ORIGINS (KARLSSON *et al*. 1997; SPICER and FRANCISCO, 1997; ZACHOW and MAGOFFIN, 1997; SPICER and FRANCISCO, 1998, AGARWAL *et al*., 1999).

In another study, we described for the first time the presence of Ob-R also in the secretory cells of the lining epithelium of the oviducts (DALL'AGLIO *et al*., 2003). By *in vitro* experiments we found that leptin inhibited PGF2α, but stimulated PGE2 release. These results give new insights over the possible role of leptin on oviductal function, because PGF2α is known to enhance smooth muscle contraction and PGE to cause relaxation (WIJAYAGUNAWARDANE *et al*., 2001). Thus, the metabolic signals carried by circulating leptin may have some role in the control of fertilisation and early embryonic development by providing a favourable local environment to gamete (sperm and oocyte) transport, sperm capacitation and oocyte maturation.

Leptin receptors have been found also in different areas of the hypothalamus and anterior pituitary of the rabbit (data not published) indicating that leptin could act at both brain and gland level to regulate gonadotropin secretion (AMSTALDEN *et al*., 2003), either directly or indirectly, pending the exact co-localization of the Ob-R within these structures. Leptin may also be involved in the control of satiety and energy metabolism through the regulation of several neurotransmitters similarly to what proposed for other species (HARRIS *et al*, 2000). In rabbits, neuropeptide Y (NPY) has been found to regulate gonadotropins release as well as feeding behaviour (KHORRAM wt al., 1988) and this finding opens new interesting perspective in the study of the relation between nutrition and control of reproductive functions.

Taken together, these observations suggest that leptin is involved in a large array of regulatory actions, whose mechanisms, however, are still poorly understood. Both the oviduct and ovarian structures can be a potential target for fine-tuned regulation by leptin, whose blood levels, reflecting whole body energy store and caloric intake, may be one of the metabolic signals which act, either centrally and/or peripherally, to switch on or off reproductive activity consequently. However, since the sequence of the gene encoding the Ob-R in rabbits is still unknown, some caution should be taken in inferring from *in vitro* results a relevant physiological significance also *in vivo*. It remains to be established, for example, which type of leptin receptors, either long or short, is indeed expressed in the ovary and the oviduct of rabbits as well as in the other tissue, including pituitary and hypothalamus, where it has been found. In fact, there are at least six Ob-R subtypes (Ob-Ra-f) arising from mRNA splice variants (HOUSEKNECHT *et al.,* 1998; SWEENEY 2002). The "long form" of the receptor (Ob-Rb) activates the janus kinase /signal transducer and activator of transcription (JAK/STAT) pathways (BAUMANN *et al*., 1996) while the "short forms" (Ob-Rc-d and Ob-Rf) trigger the MAPK pathway (BJØRBÆK *et al*., 1997; HOUSEKNECHT *et al*., 1998).

Besides leptin, however, a long list of metabolites, including glucose, NEFA and triglygerides, and hormones, such as insulin and IGF-I, are supposed to exert positive effects on ovulation rate, gonadotropins and steroid production, but, probably, no or little effect on follicle development and embryo survival.

Physiological mechanism in vivo

In different animal species, including rat, sheep, pigs, cows, primates and women, severe caloric restriction inhibits pulsatile LH secretion by depressing the GnRH pulse generator within the hypothalamus as recently reviewed by WADE *et al*. (1986) and CAMERON (1996). Interestingly, however, an increasing body of evidence suggests that even short-term changes in the level of nutrition can markedly influence reproductive function at least in some species. In primates and rats, for example, a single day of fasting is sufficient to reduce the pulsatile LH secretion while re-feeding the normal diet rapidly restores the high pulse frequency pattern of LH (SCHREIHOFER *et al*., 1993; MAEDA *et al*., 1994).

To analyse the interrelationships between nutritional factors and reproductive function, complete deprivation of food for a short period of time could be a useful experimental protocol also in rabbits. This approach has been recently applied by our group and, although research is still ongoing, there is strong evidence suggesting that nutritional challenges, evoked by one- or two-days fasting before artificial insemination (AI), have greatly influence on the gonadal axis (BONANNO *et al*., 2002). In fact, acute food deprivation markedly depressed not only sexual receptivity, fertility rate, and litter size at birth, but also reduced estradiol-17β pulse frequency and amplitude, lowered LH peak surge following GnRH injection, and modified differently the expression of oestrogen receptors (ER) in both hypothalamus and anterior pituitary (BRECCHIA *et al*., 2004).
At present, it is not clear what metabolic signals are involved in these nutritional-related effects induced by acute food deprivation and whether they act directly upon the HPO axis or indirectly to regulate the secretion of other hormones such as GH or prolactin,

which in turn may influence the gonads to reduce the release of estradiol-17β. Moreover, short-term caloric deprivation was found to regulate differently the reproductive activity of rabbits. In fact, one-day fasting reduced fertility especially on older lactating does and this finding is somewhat surprising since caloric restriction should have worsened the metabolic balance of the young does, already penalised by their physiological low feed intake (XICCATO *et al*., 2004). By contrast, when does were allowed to re-feed one day before AI, reproductive efficiency remained unaffected, suggesting that nutritional-related adverse factors are rapidly removed and that the function of HPO system is reset to its normal level.

In rabbits deprived of food for 48 hours, ER immunoreactive intensity decreased in the ventromedial nucleus, but increased in the medial preoptic area, similarly to what found in syrian hamster (LY *et al*., 1999; JONES *et al*., 2002). These data, together with the low levels of circulating estradiol-17β, may well explain the fasting-induced decrease of sexual receptivity. The low estradiol-17β plasma levels are probably due to a reduced synthesis by the ovary rather than an increased clearance by the liver, but the underlying mechanisms working *in vivo* are still unclear. The nutritional-derived factors, in fact, may target both hypothalamus and pituitary, as well as the growing follicles within the ovary through blood carried hormones, such as insulin, IGF-I and leptin, and growth factors, including TGF-β and VEGF, acting locally in a paracrine/autocrine fashion to reduce steroidogenesis. Glucocorticoid receptors have been localized in rat granulosa cells and cortisol was found to inhibit FSH-stimulated aromatase. (SCHOONMAKER and ERICKSON, 1983). Leptin was found to inhibit the production of estradiol-17β from granulose cells under different *in vitro* conditions (SPICER and FRANCISCO, 1997; AGARWAL *et al*., 1999; KARLSSON *et al*., 1997; ZACHOW and MAGOFFIN, 1997)

The LH peak surge was consistently lower in two-days fasting does than in controls or rabbits fasted for 24 hours (BRECCHIA *et al*., 2004). It remains to be established, however, which factors were responsible for the fasting-induced suppression of LH secretion, as it may be due to down regulation of pituitary GnRH receptors and/or to reduced synthesis of LH by pituitary cells. Interestingly, in pituitaries of long-term fasted does, both number and signal intensity of ER positive cells were markedly reduced compared to full-fed animals. Since steroids are know to influence the expression of several peptides related to reproductive function in the hypothalamus, the down regulation of ER within the anterior pituitary may suggest a direct physiological link between pituitary gonadotropin competence and the active status of follicles in the ovary mediated by estrogens. This hypothesis is supported, although indirectly, by the findings of CARLSON and PERRIN (1979), who observed a decreased pituitary responsiveness in GnRH-treated ovariectomized does, and by those of RODRIGUEZ *et al*. (1989), who found that the rise in plasma LH levels after GnRH administration was correlated with sexual receptivity and, thus, with circulating estrogens. The recent discovery that leptin, resistin and adiponutrin are not only expressed at gene and protein levels within the anterior pituitary, but also regulated by food restriction suggests a direct role of these pituitary-derived adipokines in the neuroendocrine regulation of energy homeostasis (WIESNER *et al*., 2004). At this moment, however, the possibility that the altered LH release detected in fasted does could be ascribed to an inhibition of the hypothalamic neural GnRH

apparatus cannot be ruled out. In sheep food deprivation failed to decrease serum LH concentrations and both GnRH receptor content and mRNA expression in the pituitary (BECKETT *et al*., 1997a; BECKETT *et al*., 1997b). In this species, during prolonged weight loss, the negative feedback action of estradiol-17β on LH pulse frequency was greatly enhanced (BECKETT *et al*., 1997b). In the rat, there is evidence that the activation of the hypothalamus-pituitary-adrenal (HPA) axis may be responsible for the fasting-induced suppression of LH secretion because administration of a corticotropin-releasing hormone antagonist reversed the hypogonadotropism arising from food deprivation (MAEDA *et al*., 1994). From our study the role of estradiol-17β in the maintenance of a functional HPO axis capable of responding appropriately to GnRH appears to be essential even if the site of its action within this system is not yet clear.

Plasma leptin concentrations were significantly lower in fasted does, but soon after re-feeding they gradually rose to match those of rabbits fed ad libitum. Similar findings were also obtained in other species (WHISNANT and HARRELL, 2002; CHELIKANI *et al*., 2004). Recently, also in heifers it has been shown that acute feed restriction markedly reduces leptin mRNA in adipose tissue and circulating concentrations of leptin (AMSTALDEN *et al*., 2000). According to MOSCHOS *et al*. (2002) leptin may act as a critical link between adipose tissue and the reproductive system, indicating whether adequate energy reserves are present for normal reproductive function. Our findings, however, suggest that leptin secretion is regulated by other factors not directly dependent by adipose mass and may act as signal on the availability of metabolic fuel in accordance with what reported also in dairy cattle (CHELIKANI *et al*., 2004).

According to CAPRIO *et al.* (2001) leptin may have a dual action on mammal reproductive function, depending on different thresholds and its site of activity. Leptin concentrations above a minimal threshold are necessary in the hypothalamus to activate the gonadal axis, for triggering puberty and maintaining normal reproductive function. Leptin excess above a certain threshold, such as found in obesity, might impair testicular and ovarian steroidogenesis and have negative effects on reproduction. In heifers, exogenous leptin administration prevented fasting-mediated reductions in the pulsatile secretion of LH and enhanced its GnRH-stimulated release (MACIEL *et al*., 2004). However, from our data there is little evidence that leptin may act in the short period of time to regulate both pituitary LH secretion and ovarian estradiol-17β production, given that in does fasted for 24 h the gonadal and pituitary responses were normal despite the severely reduced peripheral plasma leptin concentrations. Thus, it is more plausible that leptin exerts a permissive role together with other metabolic hormones, such as insulin, IGF-I and different metabolites in signalling body condition and energy reserves to the HPO for controlling the reproductive function.

Stress-related mechanisms

Normally, stress activates the hypothalamic-pituitary-adrenal (HPA) axis through the release of the corticotropin-releasing hormone (CRH). Thus, CRH is the major physiological regulator of both basal and stress-induced release of ACTH, pro-opiomelanocortin (POMC)-derived peptide, and β-endorphin, from the pituitary (De SOUZA and GRIGORIADIS, 1994). All these hormones, as well as glucocorticoids and

adrenal catecholamines that are secreted in response to generalised stress, are involved in the regulation of neural GnRH hypothalamic system and are known to exert an inhibitory action on the HPO system and reproductive processes. By converse, another POMC cleavage product, α-MSH, increases sex behaviour and LH release (GONZALEZ *et al*., 1993, GONZALEZ *et al*., 1996). But, stress activates also prolactin (PRL) release from the anterior lobe of the pituitary gland by way of the PRL-releasing factor produced in the intermediate lobe of the pituitary and in the medial basal hypothalamus (BODNAR *et al*., 2004; TORNER *et al*., 2004). Thus, besides physiological suckling episodes (MUCCIOLI *et al*., 1983; FUCHS *et al*., 1983), also environmental stress stimuli can induce increased PRL release, which may target the HPO axis and cause anestrous (KERNABON *et al*., 1994).

Rabbits appear to be very responsive, although differently, to several potentially stressful conditions and, depending on the type and intensity of the stress, either a permissive or suppressive action on the HPO axis can be observed. And stress-related mechanisms are probably implicated when rabbit does are exposed to some kind of mild stimuli of environmental or social origin such as change of cage, temporary doe-litter separation (DLS), and other bio-stimulating techniques used to improve reproductive performance as recently reviewed by THEAU-CLÉMENT (2000). In fact, although the classical response to stress usually causes a decline in FSH and LH secretions (FERIN, 1998), these "bio-stimulations", as already hypothesised by THEAU-CLÉMENT *et al*. (1998), may exert on the doe, via different neural pathways, permissive actions on the hypothalamic centres that control GnRH-dependent gonadotropins release and, indirectly, both receptivity and fertility. Therefore, these “internal” or “external” stressor inputs may target at different hypothalamic loci, both local pre-motor neurones, possibly GABAergic and glutamatergic, and GnRH neurones responsible for continuous follicular development through modulation of its tonic activity that control gonadotropin secretion from pituitary. The same stimuli may also enhance the hypothalamic inhibitory control of prolactin release by dopamina, the most powerful prolactin-inhibiting agent known. Other neuroendocrine signals are known to modulate the GnRH hypothalamic apparatus, including endogenous opioids, serotonin, and dopamine, all of which with an inhibiting role (MCCANN, 1980). Unfortunately, however, none of these substances have been proven experimentally in rabbits, either *in vivo* or *in vitro*. It is likely that the hormonal interior milieu brought over by estrogens and progestins plays a pivotal role in the expression of several neuromodulators capable of affecting hypothalamic responsiveness. Interestingly, in fact, the female rabbit has been found to respond to progestin treatments given for a short period of time with increases in GnRH spontaneous release (RAMIREZ *et al*., 1986). Thus, the possibility that during mild stress the small but significant increase in adrenal progestins that occurs in response to HPA activation (BOITI and CANALI, 1987) may enhance gonadotropin secretion cannot be ruled out.

Since the last World Rabbit Congress, few efforts have been done to study the underlying mechanisms related to the “biostimulating techniques” that may affect the HPO axis and not only the overall reproductive parameters. As far as we know, only two works by UBILLA *et al*. (2000, 2001) examined the pituitary and ovarian responses to transient doe-litter separation in nursing rabbits. They found decreased prolactin levels

24 h after DLS and increased estradiol-17β concentrations, the day of AI, and greater LH response to GnRH treatment. Accordingly, these authors suggested that the lowered prolactin concentrations, probably due to the absence of suckling episodes, may have a key role in stimulating ovarian follicular growth and steroidogenesis, and in the improvement of receptivity and fertility of does subjected to DLS. This explanation, however, might be only partially satisfactory on the ground that recent works by BONANNO *et al*. (2004) showed that the interruption of the 48-hour DLS, via controlled suckling, gave the same improvement in fertility as the regular DLS.

Whereas DLS improves the fertility of young does with less than 4 parities it is almost ineffective on older females independently of what management system is adopted (BONANNO *et al*., 1999; SZENDRO *et al*., 1999; MAERTENS, 1998; VIRAG *et al*., 1999). The possible causes for this differential age- and/or parity-dependent effect of DLS on fertility, however, have not been fully elucidated. A sparing effect on metabolic energetic resources, due to the temporary suppression of suckling associated with DLS, has been proposed by BONANNO *et al*. (1999). However, the observation that the progressive reduction of DLS efficiency was also linked to the number of repeated DLS applications on the same animal (BONANNO *et al*., 2002) supports, although indirectly, that stress-related mechanisms may be involved through a sort of progressive adaptation and/or habituation to this treatment. This speculation, however, has never been confirmed through the study of specific stress markers such as circulating catecholamines and glucocorticoids.

An interesting and unexpected side effect of the DLS technique has emerged recently when it has been observed that early neonatal maternal deprivation may exert long-term and lasting influences during postnatal development affecting endocrine, nervous and, probably, also the immune systems, as documented by the lower corticosterone response after an acute stress or ACTH inoculation, reduced mortality at weaning and improved subsequent fertility (BOITI *et al*., 2001; BRECCHIA *et al*., 2001).
Several lines of evidence suggest that exposure to different types of stress in the early stage of development may program the HPA axis differently depending on the age, due to the regulatory effects of corticosteroids upon the plasticity of neurones within several areas of the central nervous system as observed in the rat (VAN OERS *et al*., 1998). Corticosteroids are thought to be of critical importance throughout the life span. Whereas in the adult, their effects on the brain are usually reversible, during development, when neural pathways are still forming, corticosteroids may have permanent effects. Moreover, the HPA axis, through its co-ordinated actions in the nervous, endocrine and immune systems, plays a key role in the integration and regulation of the body's overall response to stress, including reproduction.

The reciprocal interactions between immune and neuroendocrine mechanisms are known since long time, but also nowadays are the subject of intensive investigations (BESEDOWSKY and DEL REY, 1996). Glucocorticoids, would favor, during ontogeny, positive selection of T lymphocytes expressing receptors with low affinity for self-antigen to become mature T cells within the thymus. Circulating glucocorticoids may contribute to the specificity of the immune response to a given antigen. By converse, a large array of immune-derived cytokines such as different classes of interleukin (IL), interferon

(IFN), and tumor necrosis factor (TNF) can affect several endocrine functions including those implied with reproduction.

Thus, the possibility of modulating positively the neuroendocrine as well as the immune system through the HPA axis during early development should not be excluded, but surely further studies are needed to confirm on a larger scale those encouraging, but indeed preliminary results, and establish both theoretical and practical bases necessary for in field application.

Luteotrophic and luteolytic mechanisms

The observation that luteal activity may affect negatively the fertility of rabbits does emerged few years ago when it was found that up to 21% of rabbit females had abnormally high plasma progesterone concentrations at the time of insemination (BOITI *et al*., 1996). These high levels of progesterone, while did not impede GnRH-induced ovulation, were indeed responsible of anti-reproductive effects. Similar findings were also reported by THEAU-CLÉMENT *et al*. (2000), who also described the presence of two populations of corpora lutea (CL) in the ovaries and complete infertility in does with progesterone above normal basal threshold. In both studies, however, the causes of these unusual findings were never identified. At least two basic mechanisms could be involved in the development of the functional CL responsible for the abnormally high blood progesterone concentrations found in rabbits during the postpartum period when it should remain at very low levels (BROWNING *et al*., 1980). The first refers to the formation of CL following spontaneous ovulation, similarly to what happens in other normally cycling species, the second to increased life span of CL due to failure or inhibition of the luteolytic mechanism. Prolonged luteal function has been observed in rabbits with induced endometritis (BOITI *et al*., 1999), thus indirectly confirming the importance of the endometrium for properly timed spontaneous luteolysis and partially explaining previous finding of the early postpartum period. It should be cautioned, however, that the assessment of those factors that might conjure in evoking abnormal luteal activity in rabbits is paved with difficulties. In fact, the high-progesterone syndrome is not uniformly distributed in rabbit farms (BOITI *et al*., 1996) so that the study of real cases is difficult to set up. Moreover, to date, no experimental design has been devised to replicate this syndrome under controlled conditions.

Catecholamines can directly or indirectly influence pituitary secretion. Whereas the principal effect of dopamine is the inhibitory action of PRL release norepinephrine has been shown t be a potent releaser of GnRH via the activation of cyclooxygenase-1 enzyme (COX-1) and PGE2 synthesis mediated by nitric oxide (NO) production (RETTORI *et al*., 1992). Oxytocin has been found to stimulate GnRH release into the hypophyseal portal vessels that mediates LH release from the pituitary (RETTORI *et al*., 1993). Thus, activation of the sympathetic nervous system by some stressful agent and/or suckling-dependent oxytocin release in particularly sensitive and estrogen-primed does may cause a LH surge sufficient to induce a spontaneous ovulation, not related, therefore, to mating or to exogenous GnRH administration.

However, another interesting possibility relies on the mounting and convergent evidence that various cytokines, such as IL-1, TNF-α, IL-6, IL-2, IFN-γ, and others, may have

direct action on the pituitary gland itself and alter pituitary hormone release and/or responsiveness to hypothalamic releasing peptides (MCCANN *et al*., 1994). Thus the possibility that bacterial invasion of the genital tract in the early postpartum days , for example, may induce, largely via lipopolysaccharide derived from the bacterial wall, the synthesis and release of cytokines which alter the neuroendocrine response of rabbit females should also be taken into consideration.

Despite an increasing amount of compelling evidence suggests the important role of estradiol-17β and PGF2α in luteal function, the underlying mechanisms that control the life span of CL are still unclear. It is evident that there are several different luteolytic and luteotrophic forces at work, besides PGF2α and estradiol-17β, PGE2, and LH. There are many levels at which these opposing and balancing influences may exert their action. The luteotrophic messenger chain links the luteotrophic receptors via G proteins to adenylate cyclase which, in turn, forms cAMP, stimulates protein kinase A, and thereby steroidogenesis. By contrast, the luteolytic messenger system leads, from the luteolytic receptor via G protein, to activation of phospholipase C, formation of IP_3 and release of calcium from sarcoplasmatic reticulum. Another luteotrophic factor is epinephrine, which probably couples to the same AC/PKA signal system as does PGE2 and LH. In rabbits, the luteolytic mechanisms come into play only after an infertile mating to reduce the length of pseudopregnancy by shortening the life span of unnecessary CL (SCOTT and RENNIE, 1970). Normally, luteolysis begins approximately on day 12 to 14 of pseudopregnancy and is completed around day 18 when progesterone declines to basal value (BROWNING *et al*., 1980). It remains to be established, however, what it is the primary cause which triggers in the rabbit the luteolytic process that, soon after its initiation, involves not only functional but also structural changes leading to the complete demise of CL (NISWENDER *et al.*, 2000). Whereas in ewes PGF2α of uterine origin has been identified as the main luteolysin factor, it is questionable whether it induces spontaneous luteolysis also in rabbits, on the ground of anatomical impairment (Del CAMPO and GINTHER, 1972) and experimental evidence. In fact, increased PGF2α levels have been found in venous blood from the uterus only around day 17 of pseudopregnancy, thus much later after the initial decline of progesterone (LYTTON and POYSER, 1982). According to GOODMAN *et al*. (1998) the initial decline in progesterone production leading to luteal regression by way of the apoptotic cascade is caused by the withdrawal of estradiol-17β action on the CL, which exerts a well-known luteotrophic effect in rabbits (HOLT, 1989).

Nevertheless, apart estradiol-17β and PGF2α, several factors are now recognised to regulate, via paracrine and/or autocrine mechanisms, the life span of CL of rabbits, from formation to regression (NISWENDER *et al*., 2000). Convincing evidence suggests that luteal NO is deeply involved in the regulation of rabbit CL function (GOBBETTI *et al*., 1999), as PGE2 exerts its luteotrophic effect through NO synthase (NOS) down-regulation, while the PGF2α luteolytic effect is mediated by NOS up-regulation both *in vitro* (BOITI *et al*., 2000; BOITI *et al*., 2002) and *in vivo* as found in luteolysis induced by exogenous administration of PGF2α (BOITI *et al.,* 2003). Both NOS isoforms, the constitutive or endothelial and the inducible, are expressed at gene and protein levels in the rabbit CL (BOITI *et al*., 2002). In the ovary and luteal tissue, endogenous NO,

besides regulating the activity of key enzymes and other transcription factors (Gow and ISCHIROPOULOS, 2001) may also inhibit 17β-estradiol synthesis (YAMAUCHI *et al.*, 1997) or stimulate luteal PGF2α release through the activation of cyclooxygenase (ESTEVEZ *et al.*, 2002). Within the CL, overproduction of NO during luteolysis may target nitration and oxidation thereby affecting specific protein function as well as causing lipid peroxidation (MOTTA *et al*., 2001), DNA damage (BECKMAN *et al*., 1990) and promote apoptosis (KIM *et al*., 1999). Thus NO is the first transmitter which, instead of acting on cell surface receptors, diffuses into the cell to activate or inhibit intracellular enzymes.

According to some recent views, luteolysis might be regarded as an immune-mediated event leading to apoptosis (TILLY, 1996), and in this context, the abnormally high concentrations of NO found during both spontaneous (BOITI *et al*., 2004) and PGF2α–induced luteolysis (BOITI *et al*., 2003) may play a major role in promoting the intracellular mechanisms of the apoptotic pathways. Probably, the cytokines secreted by resident cells or recruited immune cells normally found also in CL of rabbits (KRUSCHE *et al.*, 2001) may exert a physiological role in luteal demise by enhancing NOS activity (BOITI *et al*., 2004). In a recent study, we have demonstrated that changes in luteal p53, macrophage chemoattractant protein (MCP-1), and IL-1β gene transcripts during spontaneous luteolysis are closely coupled with the up-regulation of luteal NOS total activity and are coincident with progesterone decline (BOITI *et al*., 2004). Thus, NO may inhibit steroidogenesis and induce expression of p53 gene, a transcriptional regulator of the bcl-2 and bax genes, whose expression activates the apoptosis mechanism after removal of the protective action of progesterone, which opposes functional regression of CL. There is little doubt that complex interplays between different cytokines, due to their multiple and redundant actions, are present in the CL during regression, but It must be stressed, that a number of details are still unknown, including the exact sequence of events which, directly or indirectly, causes the up-regulation of NOS activity, and regulate the gene expression of NOS isoforms and those for cytokines. Also the actions of these cytokines on luteal function, as well as their physiological relevance and the cross talk between immune cells and luteal cells as well as the balance between pro- and anti-apoptotic cytokines demand further investigation.

Besides the involvement of immune cells in the regulation of ovarian function, increasing experimental evidence, derived by *in vivo* and *in vitro* experiments, supports the hypothesis that also other accessory cells, including the endothelial cell component, may help in regulating CL activity interacting with steroidogenic luteal cells (DEL VECCHIO and SUTHERLAN, 1997). Within the rabbit CL, the endothelial cell component represents about 30% of the overall cell population (DHARMARAJAN *et al*., 1988). Thus, endothelin–1 (ET-1), a potent vasoconstrictor synthesized by vascular endothelium, may promote the cascade mechanism of luteolysis triggered by PGF2α (MEIDAN *et al*., 1999; MILVAE, 2000). However, despite the emerging impact of this vasoactive peptide on luteal function, the underlying ET-1-dependent mechanisms that are physiologically involved in the luteolytic process in the rabbit are still unclear. Similarly, little is know about what mechanisms do protect the CL from luteolysis until day 6 of pseudopregnancy, when CL shift from complete refractoriness to partial and complete responsiveness to PGF2α treatment (BOITI *et al.* 1998) even if receptors for PGF2α are already present and

functionally linked to phospholipase C (BOITI *et al*., 2001). Preliminary results show that ET-1 induce both in vitro and in vivo luteolysis following binding to type A receptor for endothelin (ETA-R).

CONLUSIONS

It is quite evident that many questions on the mechanisms that affect the HPO axis of rabbit does are still waiting for answers. In spite of the relevant action of leptin for controlling the reproductive function of rabbits, for example, the role of other metabolic hormones and different metabolites in signalling body condition and energy reserves to the HPO remains to be elucidated.

Better knowledge of the stress-related mechanisms that influence the gonadal and adrenal axes of rabbits is of paramount importance for the understanding of basic physiological functions as well as the management and prevention of several potential stressful conditions in farm production. Nevertheless, the rabbit may well represent an ideal model for the study of the immune modulation techniques which might be applied to large farm animals.

By far, the most studied luteolytic mechanisms in rabbits are those associated to exogenous PGF2α administration for the simple reason that any change in blood hormone concentrations, any up- or down-regulation in the expression of protein and gene abundance, as a consequence of the treatment, is immediately apparent. The underlying mechanisms that control the life span of CL, however, are still unclear. Little is known about what factor effectively triggers luteolysis in normal, physiological conditions as well as what mechanisms do protect CL from luteolysis in the first days of pseudopregnancy. Further studies are necessary to understand the overall dynamic of gene and protein expression involved in the luteolytic process.

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