EFFECTS OF SHORT- AND LONG-TERM FASTING ON THE OVARIAN AXIS AND REPRODUCTIVE PERFORMANCE OF RABBITS DOES

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ABSTRACT

To assess the impact of acute nutritional challenges on reproductive performance, 116 rabbit does were randomly assigned to either "control" or two "fasting" groups. In the control group, the does were fed ad libitum (AL) while in the treated groups the animals were fasted for 24 (STF) or 48 h (LTF) before artificial insemination (AI). In both treated groups, the rabbits were re-feed 2 hours before AI, which was performed at day 11 *post partum (pp)* in STF does and at day 32 or 55 *pp* in LTF does following GnRH injection to induce ovulation. In a parallel experiment, blood samples were collected from 10 rabbits/groups by catheterization of the central ear vein at 15 min intervals for 4 h prior and after GnRH, then at hourly intervals for other 4 h, and again 24 and 48 h later to measure LH, estradiol-17β?and leptin. The expression of estradiol-17β receptors (ER) was evaluated by immunohistochemistry in the hypothalamus and anterior pituitary obtained from both control and treated rabbits after 24 and 48 h of fasting. The 24-h long fasting reduced receptivity (55.8 *vs.* 70.9%), fertility rate (42.8 *vs*. 59.2%) especially in does with parity =4 (50 *vs.* 80%; P=0.05), and kits born alive (6.6 *vs.* 7.7). Also the does fasted for 48 h, showed lower fertility (31.2 *vs.* 52,6 %) and litter size at birth (8.7 *vs.* 9.6) than controls. Following GnRH injection, the LH peak was higher in AL and STF than in LTF does (Mean ± SEM, 39.3±17.04 *vs.* 31.3±3.39 and 12.2±1.23 ng/ml, respectively). Estradiol-17β showed higher pulse frequency and amplitude in AL than in STF and LTF does. Toward the end of fasting, mean plasma leptin concentrations were lower (P≤0.01) in both STF and LTF does than in control rabbits (0.82±0.19, 1.20±0.1, and 2.32±0.33 ng/ml, respectively), but then gradually increased in both treated groups following re-alimentation. Compared to controls or STF does, ER-like immunoreactivity decreased in the ventromedial nucleus of LTF rabbits, but increased in the medial preoptic area. ER expression was greatly reduced in the anterior pituitary of LTF does.

Key words: Acute fasting, rabbit, LH, leptin, estradiol-17β, estrogen receptor.

INTRODUCTION

In commercial rabbit production, the energy deficit occurring in young rabbit does during the first lactation is, probably by far, the primary cause responsible for their poor reproductive performance (XICCATO, 1996, FORTUN-LAMOTHE, 1998). Although the causal link between nutrition and fertility is well known, precisely how caloric consumption acts on reproductive function still remains unclear. A long list of metabolites, including glucose and NEFA, and hormones, such as insulin and IGF-I, are know to regulate ovulation rate, follicle development and embryo survival (FORTUN-LAMOTHE, 2003). Recently, also leptin, an hormone produced by the adipose cells and an important component of the energy balance regulation, has been implied in several key points of the mammalian reproductive functions (CUNNINGHAM *et al*.,1999).

Severe food deprivation inhibits reproduction by acting at different levels over the hypothalamus-pituitary-ovary (HPO) axis and fasting-induced anoestrus has been reported in several animal species (DISKIN *et al*., 2003). Interestingly, an increasing body of evidence suggests that even very short-term changes in level of nutrition can markedly influence the gonadal axis (SCHREIHOFER *et al*., 1993; STEINER *et al*., 2003). Complete deprivation of food for a short period of time could be, therefore, a useful protocol for analysing the interrelationships between nutritional factors and reproductive function also in rabbits.

This study was aimed to examine the effects triggered by one- or two-days of fasting on i) the reproductive performance of rabbit does, ii) the hormonal profiles of LH and estradiol-17β, as markers of the responses of the gonadal axis, and leptin concentrations, as indicator of the energy metabolic status, as well as iii) the expression of estradiol-17β receptors (ER) in the hypothalamus and the pituitary.

MATERIALS AND METHODS

Animals

The animals were caged individually in indoor facilities under controlled environmental conditions for light (16L:8D) and temperature (18-22 °C). All animals were fed standard pellet diet ad libitum containing 11.0 MJ DE/kg dry matter and 18.3% CP, unless otherwise specified and freely watered. Does were artificially inseminated (AI) with heterospermic pool of fresh semen diluted with commercial extender following 0.8 μg GnRH analogue (Receptal Hoechst-Roussel Vet, Milan, Italy) injection. Sexual receptivity was evaluated on the day of AI by vulva colour.

Experiment 1

In two successive trials, NZW rabbit does were homogeneously assigned to control or two treated groups as follows: in the first, the does were fasted for 24 h one day before AI (STF, n = 43); in the second, the does were deprived of food for 48 h before AI (LTF,

n= 16). To the control does (n = 38 and n = 19, respectively for each trial) feed was always provided *ad libitum* (AL). Whereas LTF does and corresponding control rabbits were inseminated at day 32 or 53 *post partum (pp)*, all the others underwent AI at day 11 *pp*. Free suckling of litters equalised to 8-9 young and weaning at 31 days were standard procedure. STF and LTF does were reefed 2 h prior to AI. Sexual receptivity at AI, fertility rate, number of total born and alive-born, body weight and feed intake, litter size and weight, were measured.

The data of each trial were analysed using the GLM procedure (SAS, 1989) according to a linear model including the effect of feeding treatment (AL, fasting), parity (1-3, ≥4) and their interaction. Proportional data were considered as variable of Bernoulli (0-1). The differences between means were tested by Student "t" test.

Experiment 2

Unmated New Zealand White (HY/CR strain) rabbits (Charles River Italia, Lecco, Italy) of 5 months age, weighing 3.5-3.8 kg, were randomly assigned to one of the following three groups (10 animals/group): control (AL), short-term fasting (STF), and long-term fasting (LTF). Whereas control does were fed ad libitum, STF and LTF does were deprived of food for 24 and 48 h, respectively, before GnRH injection. From each rabbit, blood samples were collected by catheterization of the central ear vein at 15 min intervals from 4 h before to 4 h after GnRH injection, then at hourly intervals for other 4 h, and again 24 and 48 h later.

Plasma concentrations of LH were determined by a homologous double antibody RIA method. Estradiol-17β was assayed using a commercial RIA kit (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA). Leptin concentrations were determined by double antibody RIA using the multi-species leptin kit (Linco Research Inc., St. Charles, MO, USA).
The brains and the pituitaries obtained from both control and treated groups after 24 and 48 h of fasting (3 animals/group) were fixed in 4% paraformaldehyde and then embedded in paraffin. The reaction was performed on 7 µm thick serial sections using mAb anti-ER (Zymed, San Francisco, CA, USA). The reaction was then developed using avidin-biotin complex (Vector Elite Kit, Vector Laboratories, Burlingame, CA, USA) and the DAB chromogen (Vector Laboratories).

Data relative to hormone concentrations were analysed by ANOVA (SOKAL and ROHLF, 1981) for repeated measurements followed by Student's t-test. A value of $P < 0.05$ was considered significant. All statistical analyses were performed using Prism 3.0 software (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

To the best of our knowledge this is the first report to describe the effects of acute food deprivation on reproductive performance of rabbit does and its reflection on the hypothalamus-pituitary-ovary axis.

Reproductive performance. Compared to control does, STF reduced receptivity (55.8 *vs.* 70.9%), fertility rate (42.4 *vs.* 59.2%), number of total born (7.6 *vs.* 8.3) and kits born alive (6.6 *vs.* 7.7). However, the SFT-dependent effect on fertility was greatly influenced by lactation order, being significantly lower only in does with parity =4 (50 *vs.* 80%; P=0.05). Thus, the STF protocol here adopted (24-h fasting, followed by re-feeding 2 h before AI) did not improve the reproductive performance of lactating does. On the contrary, it markedly reduced fertility, especially on older does with an order of lactation ≥4 (-30 %) rather than on younger does of lower parity. This finding is somewhat surprising since the fasting, although limited to one day during the peak of lactation, should have worsened the metabolic balance of these young does, already penalised by their physiological low feed intake (Xiccato et al., 2004). The STF affected body weight of treated does which was almost 4% lower (P≤0.01) than that of full-fed control does two days later. However, soon after food deprivation, feed intake of treated does increased markedly as well as their body weight gain between days 12 and 21 of lactation so that they reached the weight of controls. Although the STF resulted in a temporary weight loss of nursed litters, as expected, successively they recovered within few days due to compensatory growth and, probably, milk production. Moreover, the 24-h fasted lactating does had lower losses of young rabbits from 10 to 31 day than control rabbits (8.6 *vs.* 13.6%; P=0.05).

The LTF negatively influenced fertility rate (31.2 *vs.* 52.6 %) and litter size at birth (8.7 *vs.* 9.6), although not significantly. The LTF program (48-h fasting, followed by re-feeding 2 hours before AI) was purposely tested to exacerbate the casual link between nutrition and reproductive function. Thus, longer food deprivation markedly decreased sexual receptivity, fertility rate and litter size at birth. Compared to both control and short-term fasting groups, it also altered hormone release by pituitary and ovary and expression of ER in pituitary tissue and hypothalamic areas.

Hormonal profiles. The energetic challenges greatly affected the temporal release patterns of estradiol-17β before and after ovulation. Estradiol-17β showed higher pulse frequency and amplitude in AL rabbits than in short-term fasted does which had longer interval between pulses. In LTF group, only one rabbit exhibited some pulses of estradiol-17β, whereas it remained constantly low in all the others.

In both control and treated groups, LH peak surged 30-60 minutes after GnRH injection (Fig. 1, left), but it was higher in AL and STF does than in LTF rabbits (Mean ± SEM, 39.3±17.04, 31.3±3.39, and 12.2±1.23 ng/ml, respectively). It remains to be established which factors are responsible for the fasting-induced suppression of LH secretion here observed, as it may be due to down regulation of pituitary GnRH receptors and/or to reduced synthesis of LH by pituitary cells. In sheep food deprivation failed to decrease

serum LH concentrations and 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).

Toward the end of fasting, mean plasma leptin concentrations were lower ($P \leq 0.01$) in both STF and LTF does than in control rabbits (0.82±0.19, 1.20±0.1, and 2.32±0.33 ng/ml, respectively,), but then gradually increased in both groups matching those of AL does within the next 4 h after re-feeding (Fig. 1, right). 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 (Moschos et al. 2002). Present 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 given that its levels were rapidly restored to control values within 1 to 2 hours after re-feeding.

ER immunolocalization: In the hypothalamus, ER immunoreactivity was evidenced with a very strong intensity especially in the ventromedial nucleus (VMN) and, with a weaker signal, also in the medial preoptic area (mPOA). In LTF rabbits, ER immunoreactivity decreased in the VMN, but increased in the mPOA in an appreciable way, similarly to what found in syrian hamster (LY *et al*., 1999; JONES *et al*., 2002). In pituitaries of LTF does, both number and signal intensity of ER positive cells were markedly reduced in comparison to full-fed animals.

The effect of feed restriction on the regulatory mechanisms of metabolism has been investigated only recently in rabbits. In this species, long-term nutrient deficiency during development has major neuro-endocrine consequences evoking prominent homeostatic reactions of the corticotropic, somatotropic, leptinergic, and thyrotropic axes, but its involvement on the gonadal axis is still unclear (ROMMERS *et al*. 2002).

The fasting-induced fall in circulating estradiol-17β is probably due to a reduced synthesis by the ovary rather than an increased clearance by the liver. The recent findings of leptin receptors in different ovarian structures of rabbits, including follicles at different stages of development, suggest that leptin may have a role in the steroidogenesis of pre- and post-ovulatory follicles (ZERANI *et al.*, 2004). Leptin receptors have been found also in the hypothalamus-anterior pituitary in the rabbit indicating that leptin could act at both brain and gland level to regulate LH secretion.

CONCLUSION

The nutritional challenges consisted in re-feeding rabbits a normal diet on the day of AI after 24 hours of fasting (STF) the day before, or 48 h (LTF) two days before.

In summary, these results suggest that short-term caloric deprivation regulate differently the reproductive activity of rabbits depending on its duration and the metabolic status

and energy reserves at the time of fasting. It also evidenced changes in circulating reproductive and metabolic hormones evoked by nutritional regimens just before ovulation as well as nutritional-dependent regulations in the expression of oestrogen receptors at the hypothalamic and pituitary levels which may be responsible for decreased sexual receptivity, fertility rate and litter size at birth. Taken together these results may contribute to get more insights on possible hormonal mechanisms mediating nutritional effects on ovulation.

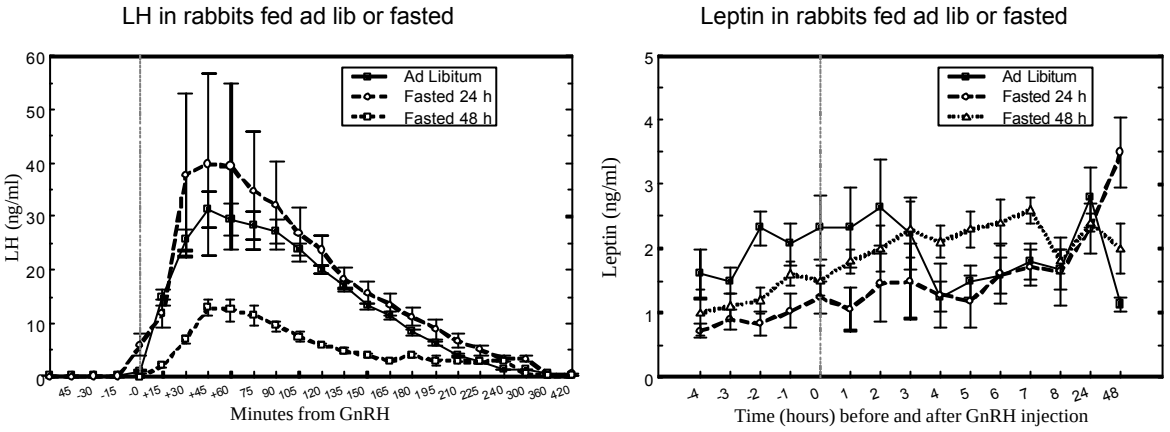

Figure 1. Plasma LH (left) and leptin (right) patterns in rabbits fed ad libitum or fasted for 24 or 48 h before GnRH challenge.

ACKNOWLEDGMENTS

We thank Dr A.F. Parlow (Harbour UCLA, Medical Centre, CA, USA) for kindling providing us rabbit LH (Lot #AFP7818C) and anti-RbLH (Lot #AFP3120489GP) reagents through NHPP program. This work was funded by MIUR (PRIN Prot. 2001072484 and 2003074002).

REFERENCES

BECKETT, J.L., SAKURAI, H., ADAMS, B. M., ADAMS, T. E. 1997a. Negative feedback potency of estradiol is increased in orchidectomized sheep during chronic nutrient deficiency. *Biol. Reprod.* **57**: 408-414.

BECKETT, J.L., SAKURAI, H., ADAMS, B. M., ADAMS, T. E. 1997b. Moderate and severe nutrient restriction has divergent effects on gonadotroph function in orchidectomized sheep. *Biol. Reprod.* **57**: 415-419.

CUNNINGHAM M.J., CLIFTON D.K., STEINER R.A. 1999. Leptin's actions on the reproductive axis: perspectives and mechanisms. *Biol. Reprod*. **60**: 216-222.

DISKIN M.G., MACKEY D.R., ROCHE J.F., SREENAN J.M. 2003. Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. *Anim. Reprod. Sci.* **78**: 345-370.

FORTUN-LAMOTHE L. 1998. Effects of pre-mating energy intake on reproductive

performance of rabbit does. *Anim. Sci.* **66**: 263-269.

FORTUN-LAMOTHE L. 2003. Bilan énergétique et gestion des réserves corporelles de la lapine: mécanismes d'action et stratégies pour améliorer la fertilité et la longévité en élevage cunicole. 2003. *10eme Journéees de la Recherche cunicole, Paris* 89-103.

JONES, J.E., PICK R.R., WADE G.N. 2002. Food deprivation inhibits estrous behaviour in hormone-treated Syrian hamsters despite elevated estradiol levels. *Hormones and Behavior* **41**: 316-320.

LI H.Y., WADE G.N., BLAUSTEIN J.D. 1999. Manipulations of metabolic fuel availability alter estrous behaviour and neuronal estrogen receptor immunoreactivity in Syrian hamsters. *Endocrinology* **135**: 240-247.

MOSCHOS S., CHAN J.L., MANTZOROS, C.S. 2002 Leptin and reproduction: a review. *Fertility and Sterility* **77**: 433-444.

ROMMERS J, BOITI C., BRECCHIA G., ZAMPINI D., MEJERHOF R., NOORDHUIZEN J.P.T.M., KEMP B. 2002. Long-term caloric restriction to ameliorate the fertility by redirection of metabolism in female rabbits. *Reprod. Domestic. Anim*. **37**(4): 249 (P1.113).

SAS, 1989. SAS/STAT User's Guide, Version 6.12. SAS Institute Inc. Cary NC. USA.

SCHREIHOFER D.A., AMICO J,A., CAMERON J.L. 1993. Reversal of fasting-induced suppression of luteinizing hormone (LH) secretion in male rhesus monkeys by intragastric nutrient infusion: evidence for rapid stimulation of LH by nutritional signals. *Endocrinology* **132**:1890-1897.

STEINER J, LAPAGLIA N., KIRSTEINS L. EMNUELE M, EMANUELE N. 2003. The response of the hypothalamic-pituitary-gonadal axis to fasting is modulated by leptin. *Endocr. Res.* **29**: 107-117.

XICCATO, G., 1996. Nutrition of lactating does. In: Proc. 6th World rabbit Congress, Toulouse, Volume 1, pp. 29-47.

ZERANI M., BOITI C., ZAMPINI D., BRECCHIA G., DELL'AGLIO C., CECCARELLI P., GOBBETTI A. 2004. Ob receptor in rabbit ovary and leptin *in vitro* regulation of corpora lutea. *J. of Endocr.* (in press).