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Hepatic Expression of Insulin-Like Growth Factor-1 in Underfed Pregnant Ewes

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Abstract: The liver is one of the most important visceral organs, which represents a large contribution to whole animal energy expenditure and the major synthetic site of insulin-like growth factor-1 (IGF-1) peptide. Decreased plane of nutrition acts by reducing the metabolic rate and mass of metabolic tissues, such as liver. Also, undernutrition results in the reduced circulating IGF-1 concentrations, due to the uncoupled growth hormone-IGF (GH-IGF) axis. This study investigated whether a 22-day period of undernutrition (half maintenance) could affect liver mass and IGF-1 protein and gene expression. Sixteen pregnant ewes fed all (n = 9) or half (n = 7) of their maintenance energy requirements were slaughtered on day 7 of pregnancy (oestrus = day 0). Body and liver mass, IGF-1 plasmatic concentrations and liver IGF-1 mRNA and protein expression were determined. Liver mass and the proportion of liver mass to empty body weight were lower in underfed animals. While IGF-1 plasmatic concentrations were lower in undernourished ewes, no differences in liver mRNA expression were found. This is the first time that differences in immunohistochemistry intensity and total content are reported in sheep. In summary, the decreased plasma IGF-1 concentrations induced by undernutrition in ewes was not associated with its reduced hepatic mRNA or protein expression, but to a decrease in liver mass.

Key words: Undernutrition, insulin-like growth factor-1, liver mass, sheep.

1. Introduction

In ruminants, visceral organs represent about 6%-10% of body weight, but they account for 40%-50% of whole body protein synthesis and heat production [1, 2]. The liver is one of the most important visceral organs, as it plays a key role in metabolism affecting efficiency of energy and nutrient utilization. Although the liver represents a small (1%-2%) proportion of animal body weight, it has a large (17%-31%) contribution to whole animal energy expenditure [1, 3]. Adaptability of ruminants to nutrient restriction depends on the capacity of their endocrine and metabolic mechanisms to maintain homeostasis [4]. It has been previously reported in several species that the decreased plane of nutrition acts at the metabolic level by reducing the metabolic rate and mass of metabolic tissues, such as liver [5, 6]. However, there were not found plenty information about dietary factors regulating visceral mass at the cellular and molecular levels in sheep. The liver is thought to be the major synthetic site of the insulin-like growth factor-1 (IGF-1) peptide [7], but also studies on knockout mice demonstrate the importance of the extrahepatic source of IGF-1, since there are evidence that liver-derived IGF-1 is not

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required for postnatal growth [8, 9]. Some immunohistochemical studies have demonstrated the existence of this peptide in the liver of mammals, such as cattle, swine and rat [10], and chicken [11], but no studies on IGF-1 protein localization in the ruminant hepatocyte were found.

During undernutrition, the growth hormone (GH)-IGF axis uncouples in the liver, which results in reduction in circulating IGF-1 concentrations, despite high GH concentrations [12]. In previous studies, it has been observed that ewes offered half of the maintenance diet (0.5 M) presented the decreased plasma IGF-1 concentrations [13], but besides the lower plasmatic concentrations of IGF-1 with undernutrition, no differences for hepatic IGF-1 mRNA expression were reported [14, 15]. Therefore, decreased circulating IGF-1 concentrations could be due to post-transcriptional mechanism (e.g., similar transcription rate but decreased protein synthesis in undernutrition), but also to a reduction in total liver mass and thus IGF-1 total content.

There are no reports showing whether or not a relation between the decreased liver mass and a reduction in IGF-1 protein exists. In this study, whether the decrease in liver mass due to undernutrition is associated with a decrease in plasma IGF-1 concentrations and if the decrease in plasma IGF-1 concentrations is a response of a diminished IGF-1 protein or gene expression in the liver were investigated.

2. Materials and Methods

The study was conducted at the Experimental Farm of the University of Zaragoza, Spain (latitude 41° N). All procedures were carried out under Project License PI05/10 approved by the in-house Ethics Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 [16] on the protection of animals used for experimental and other scientific purposes.

2.1 Experimental Design

During the breeding season in mid-October, adult Rasa Aragonesa ewes were offered a diet that supplied the daily energy and protein requirements [15] to maintain a constant body condition score (BCS) during 30 d. The diet consisted of 0.45 kg pellets and 0.55 kg barley straw per ewe per day, providing 8.37 MJ of metabolizable energy/kg dry matter and 9% of crude protein (CP). After this period, ewes were oestrus synchronized (day 0) using intravaginal sponges that contained 30 mg fluorogestone acetate (Chronogest, MSD, Madrid, Spain), which were inserted for 14 d. At the time of sponge insertion, ewes were randomly assigned into two groups to provide either 1.5 times (control nutrition: CN) or 0.5 times (low nutrition: LN) of the daily energy requirements for maintenance for 22 d. In CN group, diets consisted of 0.60 kg of pellets and 0.90 kg of barley straw (13.20 MJ metabolizable energy/ewe/day), while in LN group, diets consisted of 0.20 kg of pellets and 0.30 kg of barley straw (4.4 MJ metabolizable energy/ewe/day). The occurrence of oestrus (day 0) was checked every 8 h using vasectomized rams. All ewes were mated with intact rams.

On day 7 of the synchronized oestrus cycle, animals were euthanized using sodium thiopental (Euta-Lender; Normon SA, Madrid, Spain). Prior to slaughter, live weight (12 h of fasting) and BCS was recorded. Animals were categorized in pregnant and non-pregnant according to the presence of the embryo within the uterus, after the euthanasia. Only pregnant ewes were considered, thus the final experimental groups were: nine CN ewes and seven LN ewes. Livers were dissected and weighed. Liver samples were obtained and formalin-fixed until embedded in paraffin or snap-frozen in liquid nitrogen and stored at -80 °C until assayed. Since the weight of digest could not be registered during the sacrifice, the empty body

weight (EBW) was estimated according to equations of Cornell Net Carbohydrate and Protein System (CNCPS) for sheep [16], as Eq. (1):

$$EBW = 0.851 \times (\text{body weight} \times 0.96) \quad [1]$$

2.2 Blood Samples and Hormone Assay

Jugular blood was sampled 1 h before feeding (08:00 am) at insertion and withdrawal of the pessaries, and at euthanasia (14 d before the oestrus, day 1 and day 7, respectively). Samples were collected in heparinized tubes and centrifuged within 15 min of collection. Plasma was separated and stored at -20 °C until analysis.

Concentrations of IGF-1 were measured by a double antibody radioimmunoassay (RIA) [14]. Interference by IGF-1 binding proteins was minimized by acid-ethanol cryoprecipitation. The limit of detection was 0.49 ng/mL. All samples were analyzed in one assay, and the intra-assay coefficients of variation (CVs) for low (36.8 ng/mL) and high (520.2 ng/mL) quality controls were 1.9% and 9.1%.

2.3 Immunohistochemistry

An immunohistochemical technique (avidin-biotin-peroxidase), as previously described by Meikle et al. [17], was used to visualize IGF-1 immunostaining. Negative control was obtained by replacing the primary antibody with non-immune mouse IgG at equivalent concentration. The amount of IGF-1 was estimated subjectively by two independent observers who were not aware of treatment assignment. This peptide was evaluated in two hepatic compartments: epithelium and stoma. Ten fields were analyzed at a magnification of ×1,000 in all ewes. The staining of the hepatocyte cytoplasm was scored as negative (-), faint (1), moderate (2) or intense (3), and the extent of staining was expressed in proportion on a scale 0-10 [17]. The average staining was calculated as the method described by Boos et al. [18].

2.4 RNA Isolation and Reverse Transcription

Total RNA from liver was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), followed by precipitation with lithium chloride and DNase-treatment with DNA-Free™ Kit (Ambion, Austin, TX, USA). Concentration of RNA was determined by measuring absorbance at 260 nm, the purity of all RNA isolates was evaluated by the relation of 260 nm to 280 nm absorbance ratio and the integrity of the RNA was evaluated by electrophoresis (1% agarose gel). For each sample, cDNA was synthesized by reverse transcription using the SuperScript III transcriptase (Invitrogen) with oligo-dT primers and 1 µg total RNA added as a template.

2.5 Quantitative Real Time Polymerase Chain Reaction (PCR)

Real time PCR reactions for IGF-1 (GenBank: NM_001009774.3) and the endogenous controls hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) (GenBank: NM_001034035.2) and ribosomal protein L19 (*RPL19*) (GenBank: NM_001040516.1), were performed using 7.5 µL SYBR® Green master-mix (Quantimix EASY SYG kit, Biotools B&M Labs, Madrid, Spain), equimolar amounts of forward and reverse primers (200 nM, Operon Biotechnologies GmbH, Cologne, Germany) and 3 µL diluted cDNA (1:7.5 in RNase/DNase free water) in a final volume of 15 µL. Samples were analyzed in duplicate in a 72-disk Rotor-Gene™ 6000 (Corbett Life Sciences, Sydney, Australia). Standard amplification conditions were 3 min at 95 °C and 40 cycles of 15 s at 95 °C, 40 s at 60 °C and 10 s at 72 °C. At the end of each run, dissociation curves were analyzed to ensure that the desired amplicon was detected and to discard contaminating DNA or primer dimers. Samples of cDNA of six ewes (three of each treatment) were pooled to provide an exogenous control, and five dilutions (from 100 ng/tube to 6.25 ng/tube) of this pool were used to perform linear regression for each gene. The efficiency of the assays was calculated according to Eq. (2) [19]:

Efficiency of the assays = $(10^{-1/slope} - 1)$ (2)

Gene expression was measured by relative quantification [20] to the exogenous control and normalized to the geometric mean expression of the endogenous control genes (*HPRT* and *RPL19*), taking into account the respective efficiencies [20]. Both *HPRT* and *RPL19* have been used before as endogenous controls in liver [14], and their expression remained unchanged among samples in this study.

2.6 Statistical Analysis

All variables were analyzed by analysis of variance (SAS, Institute Inc., Cary, NC, USA) with a mixed model that included nutritional treatment (low or control) as fixed effect. In addition, the model to analyze the hepatocyte cytoplasmic staining included the effects of observer and treatment as a fixed effect, and the model for plasma IGF-1 included also the effect of the day. Data are presented as the least square means ± standard errors. Means were considered different when $P \leq 0.05$.

3. Results

Different metabolic parameters in LN and CN ewes were shown in Table 1. In LN group, body weight of euthanized ewes was 63.9 ± 0.4 kg, whereas in CN group, it was 67.3 ± 0.3 kg ($P < 0.01$). EBW of ewe was decreased by 5% in LN group, while absolute liver mass was reduced by 22% when compared with control ewes ($P < 0.01$). Liver mass relative to EBW was also reduced by 25% in LN group when compared with CN ewes ($P < 0.001$). In this study, consistent with the 25% reduction in relative liver mass, plasma IGF-1 concentrations were decreased by 24% by undernutrition ($P < 0.05$). Nevertheless, no differences were observed between ewe groups neither in hepatic IGF-1 mRNA (Table 1) nor in IGF-1 immunostaning (Fig. 1). In the present study, IGF-1 immunostaining was located exclusively in the cytoplasm of the hepatocyte.

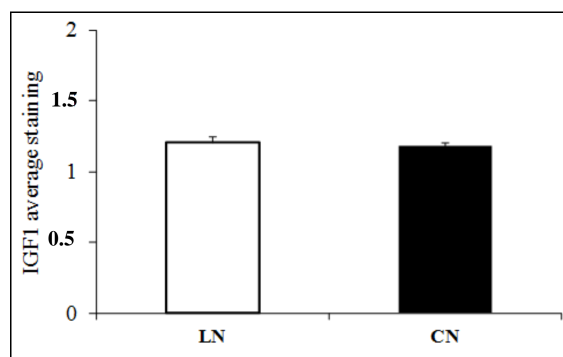
4. Discussion

The reductions in EBW and liver mass due to nutrient

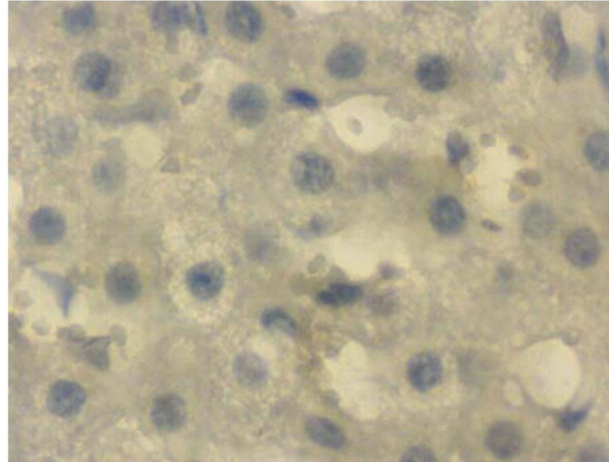
Table 1 Different metabolic parameters in LN and CN ewes on day 7 post-oestrus.

Parameters	LN (0.5 times of daily energy requirements)	CN (1.5 times of daily energy requirements)	*P* value
BW (kg)	63.9 ± 0.40	67.3 ± 0.30	< 0.01
EBW (kg)	52.2 ± 0.33	54.9 ± 0.25	< 0.01
Liver mass			
Absolute (kg)	0.63 ± 0.04	0.85 ± 0.04	< 0.01
Relative (g/kg EBW)	1.21 ± 0.07	1.54 ± 0.06	< 0.001
Plasma IGF-1 (ng/mL)	223.9 ± 25.3	295.6 ± 25.3	0.05
Hepatic IGF-1 mRNA	0.26 ± 0.04	0.31 ± 0.03	0.34

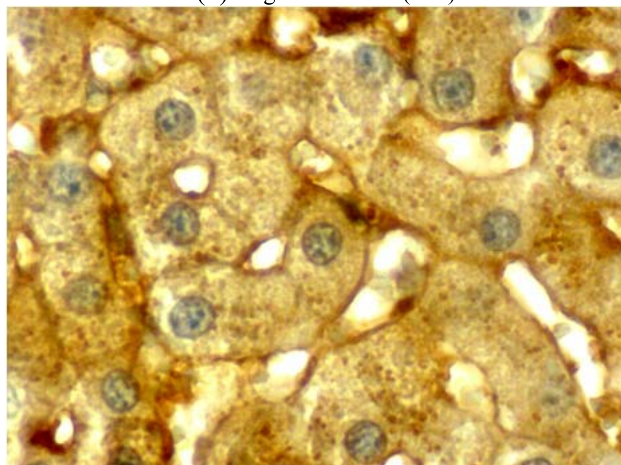
BW: body weight; EBW: empty body weight, EBW = 0.851 × (BW × 0.96).
Data are presented as least square means ± standard errors.



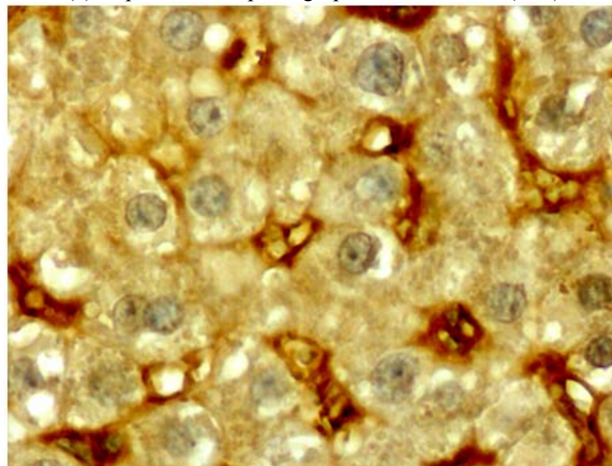
(a) Average immunoreactivity



(b) Negative control (40×)



(c) Representative photograph of control ewe (40×)



(d) Representative photograph of LN group (40×)

Fig. 1 Immunohistochemical localization of IGF-1 in liver of ewes fed 0.5 times (LN) or 1.5 times (CN) of the daily maintenance energy requirements during 22 d.

restriction are consistent with previous reports [21, 22]. In concordance with Burrin et al. [21], these findings showed that liver mass (e.g., reduced cell size and/or number) changed more rapidly than body weight in response to undernutrition, illustrating the ability of these tissues to adapt rapidly to changing

nutrient supply. In ruminants, portal blood flow is known to vary with metabolizable energy intake [1, 23], thus, in underfed animals, blood flow is reduced, leading to decreased liver nutrient absorption and in consequence a decreased liver mass.

It has been demonstrated in rats that the greatest rates of protein synthesis (e.g., IGF-1) occur in tissues, such as liver [24]. During a dietary restriction, evidence of reduced metabolic activity of the liver was apparent through less abundance of nutrient transporters. Additionally, Keogh et al. [25] observed evidence for a reduction in cell cycle processes, as well as a reduction in cellular proliferation and growth. Although the decrease in plasma IGF-1 concentrations is a mechanism of nutrient partitioning when nutrient availability is low, IGF-1 is a growth mediator that also affects the liver [26] and its decrease may lead to diminished cell number and size [27]. Although IGF-1 plasma concentrations were different between CN and LN ewes, no differences were found on hepatic IGF-1 immunoreactivity or IGF-1 mRNA. The lack of differences observed in IGF-1 mRNA between LN and CN was consistent with the previous finding by Sosa at al. [14] and those from others in beef and low milk producing dairy cows during the negative energy balance of early lactation [28]. Nevertheless, in contrast to the results in this study, Hiramatsu et al. [11] reported that fasted chicken presented lower hepatic IGF-1 immunostaining. This contradictory results could be due, among other factors, to differences in the specie (ewe vs. chicken), diets (half nutrition during 22 d vs. deprived of feed during 2 d) and immunohistochemical techniques. The absence of differences in hepatic IGF-1 immunostaning between treatments suggested that non post-transcriptional processes were responsible for the reduction found in plasma IGF-1 concentrations of undernourished ewes. In this study, it is reported for the first time in sheep that IGF-1 is localized in the hepatocyte cytoplasm, as similarly found in rodents [29] and chicken [11].

Of interest, although liver-derived IGF-I has endocrine effects on extrahepatic tissues, there are only few data regarding local effects of this hormone in the liver, probably due to the very low amount of IGF-I receptors on the hepatocytes membrane. And this would also mean that liver-derived IGF-I would be unable to stimulate liver growth [30, 31]. So the reduced liver mass found in the undernourished animals of this study may be a consequence of the reduced IGF-1 levels in serm, and would not be affected by the local transcript and protein.

5. Conclusions

These results of this study showed that the decreased liver mass in underfed ewes was associated with the decreased IGF-1 plasma concentration, and was independent of hepatic protein or mRNA expression. This suggest that plasma IGF-1 but no liver-derived IGF-1 is responsible for liver reduction mass in underfed ewes. To our knowledge, this is the first report showing the immunohistochemical localization of IGF-1 in sheep liver.

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