Glutamate supply positively affects serum cholesterol concentrations without increases in total protein and urea around the onset of puberty in goats

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**A B S T R A C T**

Different neurotransmitter and neuromodulatory systems regulate synthesis and secretion of GnRH. Whereas the endocrine and neural systems are activated in response to the metabolic status and the circulating levels of specific blood metabolites, glutamate receptors have been reported at hepatic level. This study evaluated the possible effect of glutamate supplementation upon changes in serum concentrations across time for total protein (TP), urea (UR) and cholesterol (CL) around the onset of puberty in goats. Prepuberal female goats (n = 18) were randomly assigned to: (1) excitatory amino acids group, GLUT, n = 10; 16.52 ± 1.04 kg live weight (LW), 3.4 ± 0.12 body condition score (BCS) receiving an i.v. infusion of 7 mg kg⁻¹ LW of l-glutamate, and (2) Control group, CONT, n = 8; 16.1 ± 1.04 kg LW, 3.1 ± 0.12 BCS. General averages for LW (23.2 ± 0.72 kg), BCS (3.37 ± 0.10 units), serum TP (65.28 ± 2.46 mg dL⁻¹), UR (23.42 ± 0.95 mg dL⁻¹), CL (77.89 ± 1.10 mg dL⁻¹) as well as the serum levels for TP and UR across time did not differ (P > 0.05) between treatments. However, while GLUT positively affected (P < 0.05) both the onset (207 ± 9 vs. 225 ± 12 d) and the percentage (70 vs. 25%) of females showing puberty, a treatment × time interaction effect (P < 0.05) was observed in the GLUT group, with increases in serum cholesterol, coincident with the onset of puberty. Therefore, in peripuberal glutamate supplemented goats, serum cholesterol profile could act as a metabolic modulator for the establishment of puberty, denoting also a potential role of glutamate as modulator of lipid metabolism.

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1. Introduction

In order to maximize reproductive success, the neuroendocrine system must regulate internal mechanisms to align them with respect to changes in the external environment (Scaramuzzi et al., 2006, 2011; Meza-Herrera and Tena-Sempere, 2012). First, throughout endocrine,
anatomical and physiological changes, thereafter, right through reproductive behavior. An important set of endocrinological messengers and neurotransmitters participate in such regulation (Perfito and Bentley, 2009). Nonetheless, the common initiator is GnRH whose pulsatile release pattern constitutes a key link to line up the perception of external environmental conditions with respect to the hypothalamic-hypophysal-gonadal (HHG) axis function (Perfito and Bentley, 2009; Ebling, 2009; Meza-Herrera et al., 2010, 2011). The activity and functionality of this neuronal circuitry is controlled through different neurotransmitters; activation of the complex KISS-1/kisspeptin/GPR54 system and increased glutamatergic neurotransmission are two excitatory events stimulating the onset of puberty (Maffuci and Gore, 2009; Meza-Herrera, 2009; Meza-Herrera et al., 2010; Meza-Herrera, 2012; Meza-Herrera and Tena-Sempere, 2012).

Besides to its role as the main neurotransmitter within the CNS, glutamate has also been involved in the modulation of metabolic activity, gene expression and protein synthesis (Parent et al., 2005; Meza-Herrera, 2008, 2012; Maffuci and Gore, 2009). However, reactivation of GnRH neurons at puberty not only involves changes in the expression of defined hormones or neurotransmitters (Meza-Herrera, 2008, 2012), but also can be modulated by the concentration of endogenous metabolites (Blache et al., 2006). Certainly, protein and amino acid requirements are important for growth and puberty while significant when considered within the context of body fuel utilization (Heitmann and Bergman, 1980; Meza-Herrera et al., 2004, 2007; Scaramuzzi et al., 2006, 2011; Gonzalez-Bulnes et al., 2011). Dietary low protein levels were reported to promote a reduction in body weight, exerting an inhibitory effect upon synthesis and release of LH. Therefore, serum protein level could be a key modulator of the neuronal processes involved in the increased pulsatile release pattern of GnRH observed around the onset of puberty (Meza-Herrera et al., 2007; Meza-Herrera, 2008, 2012; Gonzalez-Bulnes et al., 2011).

In addition, cholesterol, precursor of steroidal hormones, plays a fundamental role in the steroidogenic pathway which is required to promote follicular growth and development at ovarian level, in order for ovulation to occur (Gimpi and Gehring-Burger, 2007; Meza-Herrera and Tena-Sempere, 2012). Previous studies of our group demonstrated that in peri-puberal goats, glutamate acts as an important cue for sexual maturation in a glucose-independent pathway, while both T3 and insulin affected in a significant fashion the establishment of puberty in goats (Meza-Herrera et al., 2011). Nonetheless, information regarding the relative changes in blood analytes that may serve as metabolic cues reactivating the communication of the HHG axis and triggering the onset of puberty in goats, is scarce. Building on the previous study, we aimed to elucidate whether glutamate supplementation could be associated with changes in the profile of blood metabolites around the onset of puberty; the blood analytes evaluated were total protein (TP), blood urea nitrogen (UR) and total cholesterol (CL) considering to the prepuberal goat as animal model.

2. Material and methods

2.1. Location, environmental conditions, animals and feeding

The present study was conducted at the Southern Goat Research Unit, URUZA-UACH (26° 11’ NL, 103° 1’ WL, at 1117 m). Prepuberal crossbred female goats (n = 18; 3 mo. old, 7/8 Saanen-1/8 Criollo), were fed a diet to meet 100% of their nutritional requirements adjusted for live weight (LW, NRC, 1998), considering a moderate average daily gain of 50 g d⁻¹ during the 150d-experimental period. Goats were fed twice daily alfalfa hay (14% PC; 1.14 Mcal kg⁻¹ EnM) in the morning, while corn silage (8.1% PC, 1.62 EnM Mcal kg⁻¹) and corn grain (11.2% PC, 2.38 EnM Mcal kg⁻¹) in the afternoon, under natural photoperiod. During the experimental period, which included from early June to early November, goats had ad libitum access to water, shades and mineral salts. Both LW and body condition score (BCS) were recorded prior to feeding, every 30 d during the whole experimental period; BCS was determined by one experienced technician in all the animals by palpation of the goat transverse and vertical processes of the lumbar vertebrae (L2 through L5) on a five point scale (1 = emaciated, 5 = obese). All the methods used in this study were conducted in accordance to accepted international guidelines (FASS, 1999).

2.2. Experimental design, blood sampling, blood metabolites, progesterone quantification and puberty

In early June, goats (n = 18, 12.0 ± 1.2 weeks old) were randomly allocated to one of two experimental groups: (1). Excitatory amino acid (GLUT, n = 10; 16.52 ± 1.04 kg, 3.4 ± 0.12 BCS) and (2). Control, (CONT, n = 8; 16.1 ± 1.04 kg, 3.1 ± 0.12 BCS). The GLUT group received an intravenous infusion of 7 mg kg⁻¹ LW of L-glutamate twice a week throughout the experimental period (June–November); the CONT group received saline infusion. A total of 4 g of L-glutamate (Sigma Chemicals, St. Louis, MO) were dissolved in 50 mL distilled water to get a final solution for glutamate concentration of 80 mg mL⁻¹. Schedules for blood sampling collection and determination of the onset of puberty had been previously outlined (Meza-Herrera et al., 2011); the main activities will be only briefly considered. In all goats, blood samples (10 mL) were collected by jugular venipuncture, prior to feeding and twice per week from mid-June to late October. Blood samples were collected in sterile vacuum tubes (Corvac, Kendall Health care, St. Louis, MO), allowed to clot at room temperature for 30 min, and then serum was separated by centrifugation (1500 × g, 15 min), decanted and stored in duplicate in polypolyethylene microtubes at –20 °C until hormonal analysis. Blood serum progesterone concentrations were determined using components of a commercial solid-phase RIA kit (Diagnostic Products, Los Angeles, CA, USA) validated for ruminant serum (Schneider and Hallford, 1996). Intra- and inter-assay CV were 9.9% and 12.4%, respectively; average recovery was 94%. Goats with serum P₄ concentrations ≥ 1 ng mL⁻¹ in two consecutive samples were considered indicative that ovulation
had occurred and thus, as an indicator of attaining puberty (Cushwa et al., 1992); age at puberty considered the average date in which goats attained puberty within treatments.

Blood samples were collected every 2 weeks to evaluate blood metabolite concentrations; the analytes were all measured throughout spectrophotometric analyses (Coleman 15 Junior II). Serum concentration for TP was determined in duplicate by using a commercial kit based on the bicinchoninic acid reagent considering to the bovine serum albumin 16 as standard and performed as described in the manual kit (Pierce Chemical, Rockford, IL, USA). In addition, UR and CL analytes were also measured in duplicate; serum UR concentration was quantified throughout the 640-A kit, based on the urease-18 (Sigma–Aldrich Co., St. Louis, MO, USA), while serum CL concentrations were analyzed throughout the EnzyChrom™ kit (ECCH-100, Bioassay Systems, Hayward, CA, USA); assays were carried out following the protocols outlined by the manufacturer.

### 2.3. Statistical analyses

While age at puberty was compared considering an ANOVA-CRD, the proportion of goats depicting or not puberty were compared with a chi-square test. The response variables LW, BCS, serum TP, UR, and CL concentrations throughout the experimental period, were determined by split-plot ANOVA for repeated measures across time. Previously all blood analytes were log transformed because they were not normally distributed. The models included treatment in the main plot, which was tested using animal within treatment as the error term. Time and the time × treatment interaction were included in the subplot and were tested by using the residual mean square (Litell et al., 1998). In the case of a significant treatment effect, mean separations were achieved using the PDIF option of the PROC-GLM. All the analyses were computed using the GLM or FREQ procedures of the SAS statistical analysis software and were considered to be statistically significant at \( P < 0.05 \).

### Table 1

Least-square means for live weight (kg), body condition score (units), total protein (mg·dl⁻¹), urea (mg·dl⁻¹), cholesterol (mg·dl⁻¹) and puberty in goats supplemented with glutamate (GLUT) or non-supplemented (CONT) (26 North, \( n = 18 \)).

<table>
<thead>
<tr>
<th>Variables</th>
<th>GLUT</th>
<th>CONT</th>
<th>( P&gt;F )</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live weight (kg)</td>
<td>23.75</td>
<td>22.76</td>
<td>0.34</td>
<td>0.72</td>
</tr>
<tr>
<td>Body condition score (units)</td>
<td>3.69</td>
<td>3.38</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>Total protein (mg·dl⁻¹)</td>
<td>8.02</td>
<td>8.08</td>
<td>0.51</td>
<td>0.36</td>
</tr>
<tr>
<td>Urea (mg·dl⁻¹)</td>
<td>4.81</td>
<td>4.62</td>
<td>0.94</td>
<td>0.22</td>
</tr>
<tr>
<td>Cholesterol (mg·dl⁻¹)</td>
<td>77.8</td>
<td>76.9</td>
<td>0.56</td>
<td>1.10</td>
</tr>
<tr>
<td>Puberty (%)</td>
<td>70.0a</td>
<td>25.0a</td>
<td>0.05</td>
<td>36.0</td>
</tr>
<tr>
<td>Puberty (days)</td>
<td>207.0a</td>
<td>225.0a</td>
<td>0.05</td>
<td>9.2</td>
</tr>
</tbody>
</table>

\( P>F \), observed significance level.

\( * \) Average values with different superscript within line, differ \( (P < 0.05) \).

\( ^b \) Most conservative standard error is presented.

### 3. Results

Initial averages for LW and BCS were 16.65 kg and 3.31 units, with respective values at the end of the experimental period of 23.2 ± 0.72 kg and 3.37 ± 0.10. No differences \( (P > 0.05) \) between treatments were observed along the experimental period. In the same way, no differences were observed regarding neither the global serum concentrations for TP (65.28 ± 2.46 mg·dl⁻¹), and UR (23.42 ± 0.95 mg·dl⁻¹), nor those across time. Interestingly however, while a treatment × time interaction occurred between treatments for CL across time \( (P < 0.05) \), treatment differences also occurred \( (P < 0.05) \) regarding both the onset \( (207 ± 9 \text{ d vs. } 225 ± 12 \text{ d}, P < 0.05) \) and the percentage of goats depicting puberty \( (70\% \text{ vs. } 25\%, P < 0.05) \), favoring to the GLUT-supplemented group. (Table 1, Figs. 1–4).

According to the natural photoperiod observed during the experimental period, the largest photoperiod occurred in July (13.57 h) with a gradual decrease until early November (11.3 h) (Fig. 1).

### 4. Discussion

Our working hypothesis stated that glutamate administration should promote an increased level in blood

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**Fig. 1.** Reproductive outcomes of crossbred female goats (\( n = 18 \), 3 mo. old, 7/8 Alpine-Saannen 1/8 Criollo) supplemented with glutamate (GLUT) or non-supplemented, control (CONT). Arrows depict the onset of puberty (GLUT, 207 ± 9 d and CONT, 225 ± 12 d), while the right panel shows the puberty percentage (GLUT, 70% and CONT, 25%) of peripheral goats exposed to a natural decreased photoperiod [June–November, 26 h]. Note: Statistical differences \( (P < 0.05) \) were observed between treatments for both the onset and the percentage of puberty, favoring the GLUT group.
metabolites, specifically TP and COL, while a decrease in UR around the onset of puberty. According to our results, such hypothesis is only partially supported by our results, since GLUT-supplemented goats depicted increases in serum cholesterol levels across time. The last, specially towards the last 3/3 of the experimental period, coincident with the onset of puberty in the GLUT-group. Excitatory amino acids, mainly glutamate and aspartate, increase their stimulating effect upon GnRH-LH secretion during sexual maturation by increases in the release of GnRH (Urbanski and Ojeda, 1990; Van den Pol et al., 1990; Meza-Herrera, 2008, 2012). Particularly, glutamate generates a significant stimulatory effect upon the reproductive axis, especially in the establishment of the preovulatory surge of GnRH around the onset of puberty (Gore et al., 1996; Meza-Herrera et al., 2011; Meza-Herrera, 2012; Meza-Herrera and Tena-Sempere, 2012). Different neurotransmitter and neuromodulatory systems regulating both synthesis and secretion of GnRH also convey information to the CNS regarding the level of metabolic fuel as well as body energy reserves (Perfido and Bentley, 2009). Certainly, not only metabolic status but availability of nutrients are two key environmental factors required for the establishment of reproductive function (Meza-Herrera et al., 2004, 2007; Guerra-Garcia et al., 2009). The KISS-1-GPR54-kisspeptin system operates as an energetic sensor of the

Fig. 2. Serum total protein concentrations (mg dL) across time of peripuberal crossbred female goats (n = 18, 3 mo. old, 7/8 Alpine-Saanen 1/8 Criollo) supplemented with glutamate (GLUT) or non-supplemented, control (CONT). Peripuberal goats were exposed to a natural decreased photoperiod (June–November, 26 N). Note: No statistical differences (P > 0.05) were observed between treatments within month at either stage of the experimental period.

Fig. 3. Serum urea concentrations (mg dL) across time of peripuberal crossbred female goats (n = 18, 3 mo. old, 7/8 Alpine-Saanen 1/8 Criollo) supplemented with glutamate (GLUT) or non-supplemented, control (CONT). Peripuberal goats were exposed to a natural decreased photoperiod (June–November, 26 N). Note: No statistical differences (P > 0.05) were observed between treatments within month at either stage of the experimental period.

Fig. 4. Serum cholesterol concentrations (mg dL) across time of peripuberal crossbred female goats (n = 18, 3 mo. old, 7/8 Alpine-Saanen 1/8 Criollo) supplemented with glutamate (GLUT) or non-supplemented, control (CONT). Peripuberal goats were exposed to a natural decreased photoperiod (June–November, 26 N). Note: Statistical differences (P < 0.05) were observed between treatments around the last third of the experimental period coincident with the onset of puberty in the GLUT-group.
body, acting as a neuroendocrine signal that modulates the activity of the reproductive axis at puberty according to the metabolic status of the animal (Meza-Herrera et al., 2010; Meza-Herrera, 2012; Meza-Herrera and Tena-Sempere, 2012). Different studies propose that kisspeptins have been co-localized in glutamatergic neurons while have been involved in the glutamatergic control of GnRH neurons (Broman et al., 2000; Meza-Herrera et al., 2010; Hameed et al., 2011; Roa et al., 2011).

Certainly, increases in glutamatergic neurotransmission is one of the most important trans-synaptic excitatory events triggering the onset of puberty (Perfido and Bentley, 2009; Meza-Herrera et al., 2011; Meza-Herrera, 2008, 2012). The last because glutamate receptors are co-localized in several hypothalamic nuclei essential for both neuroendocrine function and activation of the Hypothalamic-Hypophyseal (H-H) axis. Although we have fragmentary knowledge regarding the mechanisms modulating the reactions controlling the intermediate metabolism (Howell et al., 2001), the earlier onset while the increased percentage of puberty observed in the GLUT-supplemented goats, suggest that such neurophysiologic scenario may involve a serum total protein/urea-independent mechanism, possibly throughout modifications in the intermediate metabolism. Glutamate is considered a key precursor particularly important in energy balance by being a primordial source of fuel during gluconeogenesis activation as well as because of its involvement in the homeostasis of protein synthesis (Wu, 1998; Howell et al., 2001; Tapiero et al., 2002).

A clear relationship has been reported among energy balance, metabolic fuel availability and reproductive outcomes. In this way, changes in the levels of blood metabolites and metabolic hormones are important signals that convey information to the CNS regarding the nutritional status of animals in order to align such metabolic status to reproductive function, activating if is the case, the HHG axis (Blache et al., 2006; Scaramuzzi et al., 2006, 2011; Meza-Herrera, 2008; Meza-Herrera and Tena-Sempere, 2012). On this respect, both the endocrine and neural systems are activated in response to the metabolic status as well as to the circulating levels of specific blood metabolites (Annison et al., 2002; Blache et al., 2006). In ruminants, in those feeding systems where energy level is limited, an increase in protein level promotes an amplification in the expression of glutamate transporters, generating an increased capability that positively influence energy status of the animal throughout an increased availability of gluconeogenic substrates.

There is compelling evidence that serum concentrations of cholesterol are closely linked to metabolic status as well as to the activation of reproductive function. In humans, significant differences have been described regarding serum cholesterol concentrations at different windows within the prepubertal-to-pubertal transition period, which was positively related to metabolic status (Pierce et al., 2010; Sorensen et al., 2010; Widen et al., 2012; Jelenkovic et al., 2013). In our study, the GLUT-supplemented goats depicted an increased percentage of puberty parallel to greater serum cholesterol concentrations across time, without differences in LW and BCS. The last suggest that besides activation of the HHG axis, glutamate supplementation also may activate other metabolic systems involved in the modulation of fuel metabolism, particularly those related to lipids. Interestingly, glutamate receptors have been reported at hepatic level, a place where cholesterol is synthesized and stored, to act, in turn, as the main precursor of steroidal hormones later involved in the activation of follicular growth and ovulation (Meza-Herrera, 2012).

Regarding the prevailing photoperiod across the study, there was observed a decrease from 13.5 to 11.3 h between July and November, generating a global decrease close to 2 h in the photic signals during the experimental period. Nonetheless, despite the GLUT-supplemented goats faced an increased photoperiod regarding the CONT-group, they depicted an earlier onset of puberty. The last suggests a positive effect of glutamate supplementation upon activation of the HHG axis while also suggests that GLUT-administration decreased the sensibility to the negative feedback that estradiol may had exerted upon the hypothalamic centers around the onset of puberty. In turn, reactivation of the HHG axis promoted the primo-ovulation in the GLUT-supplemented group (Meza-Herrera et al., 2011; Meza-Herrera, 2012; Meza-Herrera and Tena-Sempere, 2012).

Interestingly, glutamate has been also defined as the main neurotransmitter in the retinohypothalamic tract, informing to the circadian system of the CNS about the photic signal level prevailing in the external environment (Hannibal, 2002). The last is particularly to note since the reproductive consequences of puberty initiation involve the potential activation of future physiological processes highly demanding from an energetic stand of view, such as gestation and lactation. For this reason, activation of such complex neuroendocrine circuitry able to align reproductive function with respect to a changing environment, is of paramount importance to assure the reproductive success of species (Meza-Herrera and Tena-Sempere, 2012).

The current study is the first report that demonstrates that glutamate administration generates an increase in serum cholesterol concentrations across time, around the onset of puberty in the female goat. Interestingly, such physiologic scenario was no related neither to an increased live weight nor to augment body condition score. Results bring to light to total cholesterol as a key blood metabolite involved in both a precocious onset while an increased percentage of puberty considering to the goat as animal model. These results provide important information in the design of nutritional strategies to increase reproductive outputs, mainly throughout precision supplementation or focus feeding. Our findings also provide interesting information with potential translational clinical applications.

Conflict of interests

The authors report no conflict of interests and they have no direct financial relation with the commercial identities mentioned in the paper.
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