Effects of different feeding programs and ghrelin injection on plasma ghrelin concentrations and distribution of the ghrelin positive cells in the abomasum of Awassi male lambs

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SUMMARY

Ghrelin is a novel 28-amino acid peptide isolated from the rat and human stomach. This study was conducted to determine the influence of the feeding program and of ghrelin injection in lambs on the density of the ghrelin immunopositive cells in the abomasum and on the circulating ghrelin concentrations. For that, 16 Awassi male lambs, 2 month old, were allotted in 4 equal groups (group I: fed ad libitum; group II: fed ad libitum and intravenously injected with ghrelin (1 μg/kg); group III: fed once a day; group IV: fed twice a day). When lambs weighing 43 kg (around 3 month old), plasma ghrelin concentrations were measured 30 minutes before and 60 minutes after feeding by radio-immuno-assay, and after slaughtering, ghrelin positive cells were detected by immunohistochemistry (labeled avidin-biotin technique) in the cardia, fundus and pylorus regions of abomasum. Ghrelin immunoreactive cells, mainly glandular cells and some epithelial cells in the pylorus area, were scattered throughout the mucosal layer in the whole organ. The staining intensity and the density of positive cells have not significantly differed according to the stomacal zone or to the rhythm of food distribution. Furthermore, plasma ghrelin concentrations remained stable before and after feeding and although they were weakly lowered in lambs fed ad libitum, no significant difference in this parameter was evidenced between groups. These results showed that glandular cells from the abomasum expressed ghrelin and that the pattern of the peptide secretion was not closely related to the feeding program or to the ghrelin injection in lambs.

Keywords: Lamb, ghrelin, abomasum, immunohistochemistry, feeding program.

RÉSUMÉ

Effets du rythme de distribution de la ration et de l’injection de ghreline sur les concentrations plasmatiques de ghreline et sur la distribution des cellules exprimant la ghreline au sein de la caillette d’agneaux Awassi mâles

La ghreline est un peptide acide de 28 acides aminés récemment isolé de l’estomac chez l’homme et chez le rat. Cette étude a été conduite afin de déterminer l’influence du rythme d’administration de la ration et/ou d’une injection de ghreline chez l’agneau sur la densité des cellules de la caillette immunoréactives à la ghreline et sur les concentrations circulantes de ghreline. Pour cela, 16 agneaux mâles Awassi âgés de 2 mois ont été répartis en 4 groupes égaux (groupe I : nourris ad libitum, groupe II : nourris ad libitum et traités par une injection intraveineuse de ghreline (1 μg/kg)), groupe III : nourris une fois par jour, groupe IV : nourris 2 fois par jour). Lorsque les agneaux ont atteint 43 kg (à environ 3 mois), les concentrations plasmatiques de ghreline ont été mesurées par radio-immuno-essai 30 minutes avant et 60 minutes après la distribution de la ration et après euthanasie, les cellules positives à la ghreline ont été détectées par immunohistochimie (technique de marquage par l’avidine-biotine) dans les régions du cardia, du fundus et du pylore de la caillette. Les cellules immunoréactives à la ghreline, principalement glandulaires ainsi que les cellules épithéliales de la zone pylorique, étaient réparties au sein de la muqueuse sur l’ensemble de l’organe. L’intensité de coloration et la densité des cellules positives n’ont varié de façon significative ni en fonction de la zone de l’estomac, ni en fonction des modalités de distribution de la ration. De plus, les concentrations plasmatiques de ghreline sont restées relativement stables avant et après la distribution de l’aliment et, bien qu’elles fussent plus faibles chez les agneaux nourris ad libitum, aucune différence significative de ce paramètre n’a été mise en évidence entre les groupes. Ces résultats montrent que les cellules glandulaires de la caillette expriment la ghreline et que le profil de sécrétion de ce peptide n’est pas directement associé au rythme d’administration de la ration ou à un traitement par la ghreline chez l’agneau.

Mots clés : Agneau, ghreline, caillette, immunohistochimie, rythme d’administration de la ration.

Introduction

Ghrelin is a novel 28-amino acid peptide isolated from rat and human stomach. The major site of ghrelin production is the stomach mucosa whereas lower amounts are derived from the small and large intestines, pancreas, kidney, immune system, placenta, pituitary, testis, ovary and hypothalamus [4, 11, 17, 19, 29]. Ghrelin mRNA is found most abundantly in the stomach, followed by duodenum, jejunum and lung by Northern blot analysis [2]. HUANG et al. [9] reported that
ghrelin cells are mainly located in the acid-secreting mucosa, between the neck and the base of oxyntic glands, with fewer glands of the pyloric mucosa [5, 19, 32]. HAYASHIDA et al. [7] found ghrelin-immunoreactive cells to be numerous from the neck to the base of oxyntic glands in various farm animals and to be abundant in cardiac and pyloric glands in swine. YOKOYAMA et al. [39] determined that ghrelin protein was restricted to cells of the gastric mucosa and was scattered from the glandular base to the glandular neck in healthy adult male and female dogs. SAKATA et al. [21] demonstrated that ghrelin-producing cells of the rat were found in the mucosal layer of the stomach, fundus, duodenum, ileum, caecum and colon, but not in the myenteric plexus. WADA et al. [36] reported that a few ghrelin-immunopositive cells were observed in the proventriculus of the hatching chicken, but many strongly immunostained ghrelin-positive cells were scattered throughout the mucosal layer of the adult proventriculus.

Ghrelin was discovered as a peptide hormone that potently stimulates growth hormone release from the anterior pituitary, as demonstrated in rats [7, 24], humans [16, 29] and pigs [22, 23]. In addition, evidence from many species indicates that ghrelin exerts a variety of actions, affecting energy balance [15, 30], gastrointestinal motility and secretion [13, 31] and feeding behaviour [33, 37, 38]. Little is known of the role of ghrelin in the regulation of food intake and endocrine function in species other than humans and rodents. Circulating ghrelin concentrations are affected by acute and chronic changes in energy balance: they increased by fasting and decreased by feeding. The ruminant presents an interesting model for the study of ghrelin and feeding because the stomach is not emptied between periods of feeding. Despite this, there is a discernible rise in plasma ghrelin concentrations before an expected meal in sheep. The postprandial rise in plasma growth hormone (GH) levels and ghrelin secretion from the stomach may be regulated centrally through cholinergic neurons of the vague nerve [24 - 26]. HAYASHIDA et al. [7] demonstrated that plasma ghrelin concentrations in the cow are reduced 1 hour after feeding and return to pre-feeding values within 4 hours. Leaflet [12] reported that plasma ghrelin concentrations in fed steers, though considerably lower than those of fasted steers, increased just prior to feeding and diminished after feeding, whereas plasma ghrelin concentrations in fasted steers were elevated but did not seem to have a specific pattern of secretion. CLARKE et al. [3] reported that plasma ghrelin concentrations are increased pre-prandially when sheep are on a programmed feeding regimen. MIURA et al. [15] found that plasma ghrelin concentrations are affected by diurnal rhythms based on feeding schedules in mature dairy cows. Concentrations of this hormone decreased 40–60 minutes after feeding, and gradually recovered until the next feeding. Three-month-old cows have lower plasma ghrelin concentrations than adults and did not exhibit diurnal rhythms.

In this study, we first examined whether the ghrelin-immunopositive cells were localized in the abomasum of Awassi male lambs. The second aim of this study was determine the density of ghrelin immunopositive cells and the differences between plasma ghrelin concentrations under different feeding programs and ghrelin injection.

Materials and Methods

ANIMALS AND EXPERIMENTAL DESIGN

This study was conducted and validated at the Animal Welfare and Animal Production Research and Application Center in Uludağ University, Department of Veterinary Medicine (Protocol n°: 26.07.2004/020/333). Sixteen Awassi male lambs were applied to homogeneity test according to similar weight and age. The animals were 2 months old and with an average body weight of 26 kg and they were put into individual paddocks. The lambs were randomly assigned to the following 4 groups of 4 animals each according to the administration rhythm of the ration and to the ghrelin treatment: in the group I, animals were fed ad libitum, in the group II, they were also fed ad libitum and were intravenously injected with the ghrelin peptide (1 µg/kg, Ghrelin Rat, 24160 Anaspec) twice a week, in the group III, they were fed once a day (09:00) and in the group IV, the lambs were fed twice a day (09:00 and 16:00).

The daily food allowance was adjusted to metabolic energy in each day and an average body weight of 43 kg was maintained. The animals were given trefoil as dry matter. Water was available ad libitum. Concentrate feed ingredients are shown in Tables I and II. Dry matter content of dietary samples was determined by drying at 105°C for 12 hours, and crude protein content was determined by the KJELDAHL method [1]. Ash was determined by combustion at 550°C for 6 hours. The NDF (Neutral Detergent Fibre) contents were determined using the methods described by VAN SOEST et al. [35].

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Content (%)</th>
</tr>
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<tbody>
<tr>
<td>Corn grain</td>
<td>50.0</td>
</tr>
<tr>
<td>Barley</td>
<td>18.5</td>
</tr>
<tr>
<td>Sunflower meal</td>
<td>16.5</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>13.2</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.2</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin-mineral premix (^1)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

\(^1\)Vitamin-mineral premix (Kavimix VM) (supplied per kg): Vitamin A: 12 000 000 IU, Vitamin D\(3\): 3 000 000 IU, Vitamin E: 30 g, Mn: 50 g, Fe: 50 g, Zn: 50 g, Cu: 10 g, I: 0.8 g, Co: 0.1 g, Se: 0.15 g, Antioxidant: 10 g.

PLASMA GHRELIN DETERMINATION

Blood samples were obtained from the puncture of the jugular vein and collected in vacutainer tubes containing EDTA, 30 min before feeding (08:30) and 60 min after feeding (10:00) at a body weight of 43 kg for ghrelin measurements. Blood was centrifuged at 2 200g for 10 minutes at 4°C and plasma were collected and stored in microtubes containing an protease inhibitor, aprotinin (0.6 TIU (Trypsin inhibitor inhibitor...
GHRELIN IMMUNOSTAINING IN SHEEP ABOMASUM

Ghrelin-immunoreactive cells were scattered throughout the mucosal layer of the abomasum in lambs from all experimental groups. The ghrelin staining was granular and localized to the cytoplasm of glandular cells from the cardia and fundus regions whereas glandular and surface epithelial cells as well were immunopositive in the pylorus region. The labelling was particularly intense in the perinuclear cytoplasm of positive cells from the cardia region (figure 1), in the apical pole in positive cells from the fundus region (figure 2) and in the perinuclear cytoplasm and in nucleus in positive cells from the pylorus region (figure 3). Negative controls did not give any specific immunostaining for ghrelin.

The reaction intensity (staining intensity) observed in the ghrelin immunopositive cells in the different parts of the abomasum was maximal in lambs fed ad libitum and treated with the exogenous ghrelin (group II) and the histochemical parameter was minimal in lambs fed twice a day (group IV). Nevertheless, the immunostaining intensity has not significantly varied between the four experimental groups. Whatever the rhythm of food administration, the mean reaction intensity was lower in the pylorus region than in the other stomacal regions but differences were not statistically significant (Table III). Globally, the stomacal density of the ghrelin positive cells has not significantly differed between the experimental groups although it appeared weakly depressed in lambs fed twice a day (group IV) (Table IV).

However, the repartition of the positive immunolabelled cells has slightly, but not significantly, fluctuated in the three parts of the stomach according to the rhythm of food administration: the ghrelin immunopositive cells were more abundant in the cardia region than in other parts of the abomasum in lambs fed ad libitum and treated with ghrelin (group II) or fed once a day (group III) whereas the positive cell density was maximal in the pylorus region in lambs fed ad libitum (group I) and it was roughly similar in the 3 parts of the abomasum in the group IV (lambs fed twice a day). There was no statistical relationship between the ghrelin-positive cell density and the reaction intensity.

PLASMA GHRELIN CONCENTRATIONS

Plasma ghrelin concentrations 30 minutes before feeding and 60 minutes after feeding were not statistically different.
whatever the rhythm of food administration. Moreover, there was no significant difference in the peptide concentrations between groups although plasma ghrelin concentrations were lower in the group fed ad libitum (group I) than in the 3 other experimental groups (Table V).

Discussion

Since ghrelin was identified in 1999, it has been intensely studied, with most attention paid to its physiological and biochemical properties. The major site of ghrelin production in rodents and humans is the stomach mucosa [2, 4, 11, 17, 20, 21, 29, 31]. ARIYASU et al. [2] reported that ghrelin mRNA existed in the stomach of humans, while HUANG et al. [9] observed ghrelin mRNA in the abomasum of sheep. As reported in the literature [5, 7, 18-20, 32, 36, 39], ghrelin positive cells are scattered throughout the mucosal layer in all of the regions of the glandular stomach in chickens and in mammalian species. In agreement, in the present study, ghrelin was expressed by glandular cells from the whole abomasum (cardia, fundus and pylorus) in lambs and especially in the surface epithelial cells in the pylorus region. GOVONI et al. [6] observed by immunohistochemistry that there was no

<table>
<thead>
<tr>
<th></th>
<th>Cardia</th>
<th>Fundus</th>
<th>Pylorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (ad libitum)</td>
<td>1.39 ± 0.07</td>
<td>1.37 ± 0.06</td>
<td>1.03 ± 0.17</td>
</tr>
<tr>
<td>Group II (ad libitum + ghrelin)</td>
<td>1.88 ± 0.32</td>
<td>1.91 ± 0.03</td>
<td>1.35 ± 0.32</td>
</tr>
<tr>
<td>Group III (fed once a day)</td>
<td>1.62 ± 0.27</td>
<td>1.40 ± 0.25</td>
<td>1.19 ± 0.19</td>
</tr>
<tr>
<td>Group IV (fed twice a day)</td>
<td>0.87 ± 0.14</td>
<td>1.28 ± 0.03</td>
<td>0.83 ± 0.13</td>
</tr>
</tbody>
</table>

**Table III:** Reaction intensity (1+, 2+, 3+) of the ghrelin immunopositive cells found in abomasum (cardia, fundus, pylorus) of lambs weighing 43 kg and fed ad libitum (group I), fed ad libitum and treated with exogenous ghrelin (1µg/kg) (group II), fed once a day at 09:00 (group III) and fed twice a day at 09:00 and 16:00 (group IV) (n = 4 in each group). Results are expressed as mean ± SEM (standard error of the mean) (1 slide from each abomasum region of every lamb were analysed by immunohistochemistry).
clear change in gastric ghrelin immunoreactive cells in prepubertal pigs after starvation and refeeding. Our results are consistent with this work since the density of the ghrelin-immunopositive cells and the immunolabelling intensity in the three abomasal regions were not different between the experimental groups. However, the ghrelin surge occurring just before each meal in sheep on a restricted feeding regimen, whereas no significant change was found in sheep feed ad libitum. Our results are consistent with those reports: in the current study, lower ghrelin concentrations than in the other experimental groups were found in lambs fed ad libitum. But, on the other hand, plasma ghrelin fluctuations were not observed in programmed feeding groups before and after feeding. The relatively constant level of plasma ghrelin agrees with the findings of IQBAL et al. [10] who reported that voluntary food intake in a ruminant species is not influenced by central administration of ghrelin. In the same way, MELENDEZ et al. [14] also reported that there was no relation between ghrelin concentrations and feeding in sheep. However, HAYASHIDA et al. [7] and Leaflet [12] observed that serum ghrelin concentrations increased during fasting and were reduced by refeeding in ad libitum-fed Japanese black cattle and beef cattle but they failed to evidence a specific pattern of serum ghrelin concentrations. Additionally, MIURA et al. [15] found that plasma ghrelin concentrations in calves were lower than in adults and did not respond to feeding in contrast to the adult cows. Thus, the ghrelin secretory system may be immature in 3-month old calves. This observation provides another dimension to the relationship between feeding and ghrelin secretion. As the Awassi male lambs used in this study were still maturing during the experimental period, they could adapt to the different feeding regimens during this period, and consequently, the circulating ghrelin concentrations were relatively close between the experimental groups.

The sheep in this study were on programmed feeding regimens, that preserving the ghrelin preprandial secretion. These results indicate that ghrelin cannot be considered as a significant regulator of feeding behaviour in this species but

<table>
<thead>
<tr>
<th>Ghrelin immunpositive cell density (cells / mm²)</th>
<th>Cardia</th>
<th>Fundus</th>
<th>Pylorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (ad libitum)</td>
<td>19.70 ± 6.80</td>
<td>25.37 ± 3.40</td>
<td>39.60 ± 5.56</td>
</tr>
<tr>
<td>Group II (ad libitum + ghrelin)</td>
<td>35.50 ± 5.56</td>
<td>18.45 ± 5.56</td>
<td>29.60 ± 3.41</td>
</tr>
<tr>
<td>Group III (fed once a day)</td>
<td>40.60 ± 4.82</td>
<td>22.05 ± 3.93</td>
<td>22.80 ± 3.40</td>
</tr>
<tr>
<td>Group IV (fed twice a day)</td>
<td>17.25 ± 6.81</td>
<td>15.50 ± 1.96</td>
<td>14.90 ± 1.96</td>
</tr>
</tbody>
</table>

**TABLE IV:** Density (cells / mm²) of ghrelin immunpositive cell found in abomasum (cardia, fundus, pylorus) of lambs weighing 43 kg and fed ad libitum (group I), fed ad libitum and treated with exogenous ghrelin (1µg/kg) (group II), fed once a day at 09:00 (group III) and fed twice a day at 09:00 and 16:00 (group IV) (n = 4 in each group). Results are expressed as mean ± SEM (standard error of the mean) (1 slide from each abomasum region of every lamb were analysed by immunohistochemistry).

<table>
<thead>
<tr>
<th>Before feeding (30 minutes)</th>
<th>After feeding (60 minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (ad libitum)</td>
<td>5.61 ± 0.44</td>
</tr>
<tr>
<td>Group II (ad libitum + ghrelin)</td>
<td>6.61 ± 0.52</td>
</tr>
<tr>
<td>Group III (fed once a day)</td>
<td>6.26 ± 2.00</td>
</tr>
<tr>
<td>Group IV (fed twice a day)</td>
<td>6.27 ± 2.21</td>
</tr>
</tbody>
</table>

**TABLE V:** Plasma ghrelin concentrations (ng/L) before and after the food distribution in lambs weighing 43 kg and fed ad libitum (group I), fed ad libitum and treated with exogenous ghrelin (1µg/kg) (group II), fed once a day at 09:00 (group III) and fed twice a day at 09:00 and 16:00 (group IV) (n = 4 in each group). Results are expressed as mean ± SEM (standard error of the mean).
might be indirectly regulated by central and peripheral factors. In conclusion, this work showed the abomasal localization of ghrelin and growth hormone concentrations in mature Holstein cows and three-month-old calves. J. Anim. Sci., 2004, 82, 1329-1333.


