

Central estrogen-like effect of genistein on growth hormone secretion in the ewe

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Abstract. The present study tested a hypothesis, whether plant-derived genistein influences the secretion of growth hormone (GH) in ewes, acting directly within the central nervous system (CNS). Starting six weeks after ovariectomy, ewes were infused intracerebroventricularly with genistein ($n=5$) or 17β -estradiol ($n=5$), both in a total dose of 40 $\mu\text{g}/400\text{ }\mu\text{l}/4\text{ h}$, or with a vehicle (control, $n=5$). All infusions were performed from 10:00 AM to 2:00 PM and blood samples were collected from 8:00 AM to 8:00 PM at 10-min intervals. Five genistein- and three vehicle-infused ewes were slaughtered the following morning. The plasma GH concentration was assayed by the radioimmunoassay method, and immunoreactivity of GH in the adenohypophysis was determined by immunohistochemistry. In genistein-infused ewes, mean plasma GH concentration was significantly higher during the whole period of infusion than the concomitant concentration in vehicle-infused ewes. However, examining data within group, GH secretion rose gradually, reaching a significant value during the second phase of genistein infusion. In 17β -estradiol-infused animals, a significant increase in GH concentration was noted during the first two hours of the infusion, in comparison with vehicle-infused and also in comparison with genistein-infused ewes. Although a gradual increase in basic GH secretion continued in all treated groups during the afternoon and evening, mean plasma GH concentrations in genistein- and 17β -estradiol-infused ewes were still significantly higher than in the vehicle-infused. The percentage of GH-positive cells in the adenohypophysis and the density of immunoreactive material in these cells decreased significantly in genistein-infused ewes, compared to the control, indicating diminished hormone storage. In conclusion, genistein as 17β -estradiol, is an effective stimulator of GH secretion in ewes and may exert its effect at the level of the CNS.

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INTRODUCTION

Genistein belongs to a family of isoflavone phytoestrogens, which are found in many plants that are used in ruminant feeds (Adams 1995). Although phytoestrogens are partially metabolized by ruminal microorganisms after ingestion, they have been detected in constitutional fluids, i.e. blood plasma and milk, as well as in numerous animal tissues, including the reproductive organs and brain (King et al. 1998, Lundh et al. 1990). The chemical structure of isoflavones and their metabolites is similar to estrogens enabling them to bind to estrogen receptor (ER) α and β (Kuiper et al. 1997, 1998); thus they are capable of evoking typical estrogenic responses and/or have the potential to exert antagonistic effects (Cassidy and Faughnan 2000). The effects of biological activity of phytoestrogens in ewes were widely described as reproductive disturbances (Adams 1977, Firth et al. 1977, Newsome and Kitts 1977), which in part may result from the action of these compounds at the level of the central nervous system (CNS) and/or pituitary gland as well (Polkowska et al. 2004, Wójcik-Gładysz et al. 2005).

The secretion of growth hormone (GH) from the anterior pituitary is primarily under bimodal control of the hypothalamic neuropeptides, growth hormone-releasing hormone (GHRH) and somatostatin (SS). Changes in circulating levels of sex steroids may modulate the synthesis, content and release of GH from the somatotropes, which could be due to direct action of gonadal steroids on the pituitary cells, or *via* modulation of these hypothalamic neuropeptides (Jansson et al. 1984, Martinoli et al. 1991). A study by Scanlan and Skinner (2002) revealed that in ewes only a few GH cells contained ER, suggesting that any direct modulatory effect of estrogens on the somatotropes, through the ER, could be minimal. Although in rat, evidence exists that GHRH neurons are direct target cells for estrogens (Kamegai et al. 2001, Schimizu et al. 2005), both neuropeptide systems known to control GH secretion are activated in response to estrogen treatment in ewes (Scanlan et al. 2003), indicating a more complicated mechanism in this species. Based on these data, the present study was designed to test whether the plant-derived isoflavone, genistein, could influence the secretion of GH in ovariectomized (OVX) ewes, when administered directly into the third cerebral ventricle. Its effect was also compared to the effect of 17 β -estradiol, administered in the same dose and manner.

METHODS

Animals and management

All animal procedures were conducted in compliance with the Polish Guide for the Care and Use of Animals (1997) and approved by the Local Ethics Committee. Fifteen adult Polish Lowland ewes were used in the experiment. The animals were maintained indoors under natural lighting conditions (52° N, 21° E) and fed a constant diet of commercial concentrates, with hay and water available *ad libitum*. All ewes were ovariectomized six weeks before the experiment, to remove the main source of endogenous estrogens and after three weeks implanted with a stainless steel guide cannula (1.2 mm o.d.) into the third ventricle. The implantation was performed under general anesthesia, through a drill hole in the skull, according to the stereotaxic co-ordinate system for sheep hypothalamus (Welento et al. 1969) and the procedure described by Traczyk and Przekop (1963). The guide cannula was fixed to the skull with stainless steel screws and dental cement. The external opening to the canal was closed with a stainless steel cap. The placement of the guide cannula was confirmed by the outflow of a small amount of cerebro-spinal fluid during surgery and after slaughtering.

Experimental procedure

The experiment was performed during a period of shortening day length, from mid October to mid December. Starting three weeks after the cannula implantation, ewes were randomly divided into three groups and infused intracerebroventricularly (icv) with genistein ($n=5$), 17 β -estradiol ($n=5$) and vehicle solution (control, $n=5$). Genistein (Sigma) and 17 β -estradiol (Sigma) were dissolved in ethanol and stored at -20°C as stock solutions (1 mg/500 μ l) for no longer than three days. They were dissolved in saline immediately before administration. A similar solution without estrogens was prepared as a vehicle. Genistein and 17 β -estradiol were infused in the same dose (10 μ g/100 μ l/h, total 40 μ g), from 10:00 AM to 2:00 PM, using a BAS Bee™ microinjection pump (Bioanalytical Systems Inc., USA) and calibrated 1.0-ml gas-tight syringes. The dose of genistein was selected according to our previous study, in which it evoked significant changes in the secretory activity of

the gonadotropic axis (Wójcik-Gładysz et al. 2005). Blood samples were collected from 8:00 AM to 8:00 PM, at 10 minutes intervals through a catheter inserted into the jugular vein a day before the experiment. Blood volume taken each time was about 4 ml per sample, i.e., about 5% of the total blood volume was withdrawn during the collection period. After centrifugation in heparinized tubes, plasma was stored at -20°C until GH assay was performed. During the experiments, the animals were kept in comfortable cages where they could lie down and had unrestrained access to hay and water. Five genistein-infused ewes and three vehicle-infused ewes were slaughtered 12 h after the end of the experiment (next morning) by decapitation under pentobarbital anaesthesia in the local licensed slaughterhouse.

Tissue preparation

Immediately after decapitation, brains were perfused with 2 000 ml 0.1 M phosphate buffered saline (PBS; Sigma, St Louis, USA) and subsequently with 2000 ml 0.1 M PBS containing 4% (w/v) paraformaldehyde (Sigma-Aldrich, Seelze, Germany) and 15% saturated picric acid (MERCK, Darmstadt, Germany) solution (w/v), pH 7.4, *via* both carotid arteries. The hypophyses were dissected 20 min after the beginning of perfusion and postfixed for 48 h by immersion in the same fixative and washed with 0.01 M PBS. The tissues were dehydrated in graded series of ethanol, embedded in paraplast (Sigma, St Louis, USA) and 4 μm sections were cut in the sagittal plane.

Immunohistochemistry (IHC)

Immunohistochemical detection of GH in morphologically preserved pituitary sections was performed by the peroxidase labelled antibody method of Nakane and Pierce (1966). Sections were incubated with anti-hGH (ref. 19558) primary antisera diluted 1:2000 for 48 h at 4°C . Methodological details of their preparation and their specificity were described by Dubois (1971). After that, sections were incubated for 2 h at room temperature with the secondary antibody (sheep anti-rabbit Ig [H + L] labelled with peroxidase; BIO-RAD, Steenvoorde, France) diluted 1:40. For control staining, the primary antiserum was replaced with the same dilution of rabbit serum. To evaluate the specificity of staining for GH, antisera were preadsorbed using syn-

thetic hGH (Sigma, St Louis, USA). hGH antiserum was mixed with 4 mg/ml synthetic antigen, preincubated for 24 h at 4°C and then used for the IHC staining instead of the primary antibody. Neither of the controls exhibited any specific staining (data not shown).

Image analysis

A type 104 Nikon projection microscope (Nikon Corporation, Yokohama, Japan) was used for image analyses of the pituitary sections. The staining was analyzed using the Lucia image analysis computer system version 3.51ab (Laboratory Imaging Ltd, Prague, Czech Republic). Analyses were performed under a 40x objective. Pictures of stained sections were taken with a camera (Panasonic KR222, Matsushita Electric Industrial Co., Osaka, Japan) and projected on a color monitor. Images were adjusted for optimal contrast, fixed at the same brightness level, converted to gray and processed by background subtraction and removal of artifacts. Two parameters were measured to characterize the adenohypophyseal cells expressing ir GH: (i) the area fraction (percentage of total area that exhibited positive staining, indicated the percent of stained cells) and (ii) the integral density (the sum of individual optical densities of each pixel in the area being measured, indicated the amount of substance in tissue sections, expressed in relative units). Quantitative analyses were performed for each pituitary in subareas of pars distalis using a threshold function to select a range of gray values that were identified as positive staining. The immunoreaction and/or *in situ* hybridization signal in somatotropes were analyzed in four sections of each adenohypophysis, using every 40th section (16 fields of 0.0837 mm² measured in each section). The frame size was kept constant for the duration of the image analysis.

Plasma GH assay

Plasma GH concentration was assayed in duplicate by double-antibody RIA method, according to Slaba and coauthors (1994) and Wójcik-Gładysz and others (2002). The pituitary-derived bovine GH, purified by chromatography on SE-Sephadex C-50 in sodium acetate buffer was used for iodination and reference standards. The assay sensitivity for GH was 0.68 ng/ml, and the intra- and interassay coefficients of variation were 5.9% and 10.2%, respectively.

Statistics

Plasma GH concentrations are expressed as a means \pm SEM. The treatments' effects on GH concentrations were analyzed by the one-way analysis of variation (ANOVA) followed by the least significant difference test (STATISTICA). To precisely express the dynamics of changes in GH secretion in response to genistein and 17 β -estradiol, the statistical analysis of the data included similar 2-hour periods.

The quantitative measurements taken from each section of each pituitary were averaged to obtain a mean estimate for the pituitary of each animal. Then, the mean data were pooled to represent the individual treatment groups. Thus, the data are reported as the mean percentage (for vehicle-infused ewes $n=3$, and for genistein-infused ewes $n=5$) \pm SEM of the total area that exhibited positive and specific immunostaining and mean relative units \pm SEM of the integral density, according to each group. Significant effects of genistein infusion on the factors studied were assessed by the Student's *t*-test (STATISTICA). Significance for IHC was defined at the $P<0.001$ level.

RESULTS

Plasma GH concentration

Similar levels of GH secretion were observed before the infusions in all treatment groups (Fig. 1). In vehicle-infused ewes, a significant increase in GH concentration was observed after the infusion, compared to the values recorded before and during it ($P<0.05$ – $P<0.01$). In genistein-infused ewes, GH concentration increased significantly during the last two hours of the infusion, compared to the pre-infusion period ($P<0.01$) and reached the maximum value after the infusion ($P<0.001$). In 17 β -estradiol-infused animals, plasma GH concentration increased significantly during the first two hours of the infusion, compared to the pre-infusion period ($P<0.01$) and the second increase was recorded during the evening hours ($P<0.001$). Specifically, the response of GH to infused estrogens comprised of one or several pulses of high amplitude and varied individually.

Statistical comparisons between groups revealed that in genistein-infused ewes, mean plasma GH concentration was significantly higher during the entire 4-hour period of the infusion than the concomitant

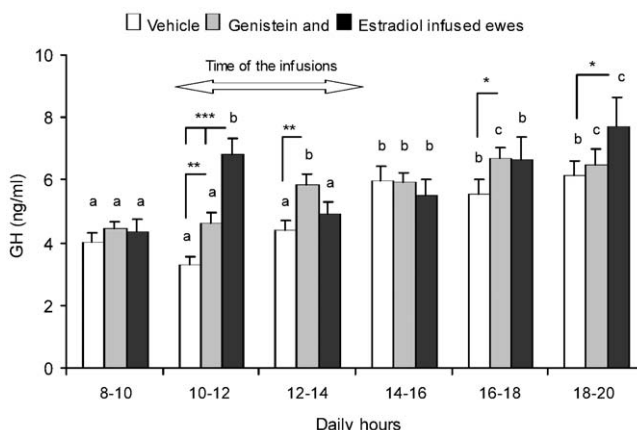


Fig. 1. Mean (\pm SEM) plasma concentrations of growth hormone (GH) in vehicle-, genistein- and 17 β -estradiol-infused ewes during 2-hour periods. All infusions were performed from 10:00 AM to 2:00 PM (white arrow). Sunset at 4:00 PM. Significant differences in GH concentrations between particular periods within groups are indicated with different letters: $a<b<c$, same letters indicate no significant differences. Significant differences between groups in particular periods are indicated by asterisks: * $P<0.05$; ** $P<0.01$ and *** $P<0.001$.

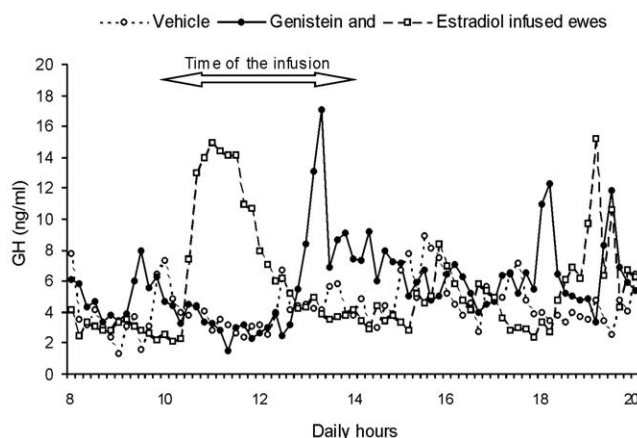


Fig. 2. Representative patterns of GH secretion in vehicle-, genistein- and 17 β -estradiol-infused ewes. All infusions were performed from 10:00 AM to 2:00 PM (white arrow). Sunset at 4:00 PM.

concentration in vehicle-infused ewes ($P<0.01$, Fig. 1). In 17 β -estradiol-infused animals, a significant increase in GH concentration was noted during the first two hours of the infusion, in comparison with vehicle-infused ($P<0.001$) and also in comparison with genistein-infused ewes ($P<0.001$). Although a gradual increase in basic GH secretion continued in all treatment groups during the afternoon and evening,

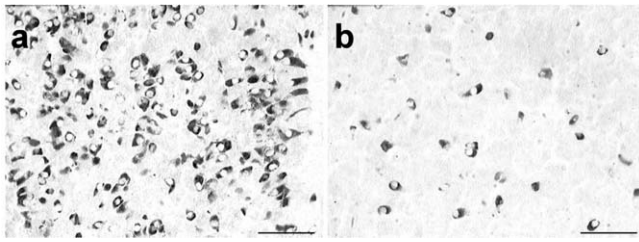


Fig. 3. Adenohypophyseal somatotropes containing immunoreactive (ir) GH in the representative ovariectomized sheep after vehicle (a) and genistein (b) infusions into the third ventricle of the brain. Note the lower population of ir somatotropes in sheep after genistein infusion in comparison with vehicle-infused sheep. Calibration bars are 50 μ m.

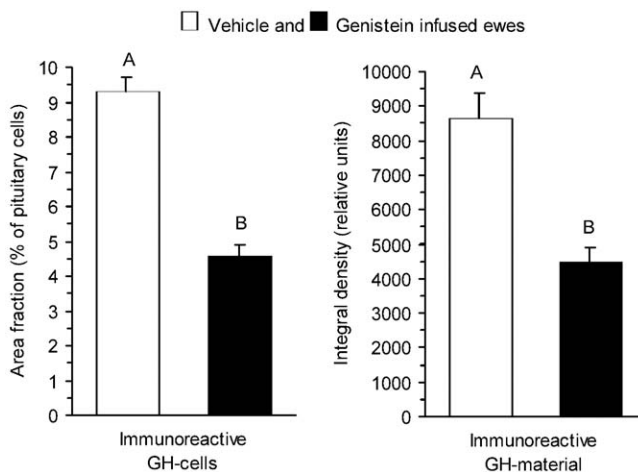


Fig. 4. The percentage of adenohypophyseal area occupied by immunoreactive (ir) somatotropes expressed by area fraction (%), and integral density (relative units) for ir GH in ovariectomized sheep after vehicle (white bars) and genistein (black bars) infusions into the third ventricle. Values are mean \pm SEM; AB, $P < 0.001$ (t -Test).

mean plasma GH concentrations in genistein- and 17β -estradiol-infused ewes were still significantly higher than in the vehicle-infused ($P < 0.05$). Examples of individual patterns of GH secretion in all treatment groups are shown in Fig. 2.

Immunohistochemistry

The microscopic observations revealed a lower number of immuno-positive GH cells and lower intensity of the staining in genistein-infused ewes in comparison with those vehicle-infused (Fig. 3). This was reflected in the results of image analyses related to the significant decrease in the percentage of adenohy-

pophyseal area occupied by ir GH-positive cells and in the density of the ir material within the pituitaries of genistein-treated ewes in contrast to vehicle-treated ewes ($P < 0.001$; Fig. 4).

DISCUSSION

The study demonstrated that genistein, a plant-derived isoflavone, might be a potent stimulator of GH secretion in the ewe. It was reflected both by increased plasma GH concentration during the icv genistein infusion and by distinctly diminished storage of GH in the pituitary somatotropes, the effect observed several hours later. The changes in the pattern of GH secretion in response to genistein were similar to that evoked by 17β -estradiol.

Numerous studies demonstrated close relationships between the endogenous gonadal steroids and the somatotrophic activity both in women and different female animal species. Faria and coauthors (1992) demonstrated the 2-fold elevation of GH in the late follicular phase of the normal menstrual cycle in women. Ovesen and others (1998), in turn, showed an increase in the frequency and amplitude of GH pulses during the preovulatory phase concomitantly with a rise in serum estradiol. Similar cyclic changes in GH secretion have been reported for small ruminants, sheep and goats (Landefeld and Suttie 1989, Yonezawa et al. 2005). There was a significant rise in both ovine GH messenger ribonucleic acid (mRNA) and serum GH concentration during the follicular phase of the estrous cycle, occurring near the peak of the luteinizing hormone (LH) surge (Landefeld and Suttie 1989). Moreover, in OVX or intact anestrus ewes, goats and in primates, estradiol stimulated increases in GH and also in insulin-like growth factor-1 (Copeland et al. 1984, Landefeld and Suttie 1989, Scanlan and Skinner 2002, Yonezawa et al. 2005). In our present study, exogenous estradiol infused into the third ventricle of the brain resulted in a marked increase in the plasma GH concentration. The elevated GH secretion, manifested by pulses of high amplitude, was maintained in 4 of 5 animals to the end of the experiment. As expected, a similar GH response was observed as a result of genistein infusion in all treated ewes, however, examining data within the group, GH secretion rose gradually, reaching the significant value during the second phase of genistein infusion. It might in part reflect a lower affinity of genistein for ERs, compared with

estradiol (Kuiper et al. 1997, 1998). Interestingly, episodic increases in plasma GH concentration were also observed in control animals during the post-infusion period. Although it could be due to the diurnal rhythm in GH secretion, as it was also observed in male sheep around the time of sunset (Romanowicz et al. 2004), one could expect a relationship between the decreased blood volume and increased plasma GH level, resulting from the multiple bleeding. Evidence exists that GH affects positively the distribution of body fluids and production of blood cells in human (Christ et al. 1997, Hanley et al. 2005). To strengthen our conviction concerning genistein action, there are changes in the number of somatotropes stained by IHC and their lowered ir-hormonal content, which may be interpreted as a decrease in hormone storage. Thus, we have demonstrated that in OVX ewes, icv. infusion of genistein results in increased somatotrope releasing activity, which is manifested by distinct diminution in the storage of GH and by the increase in plasma GH concentration. In confirmation of the estrogen-like effect of genistein it is worth noting that similar icv. infusion of this isoflavone stimulated activity of the gonadotropin-releasing hormone/LH axis in anestrus and OVX ewes (Polkowska et al. 2004, Wójcik-Gładysz et al. 2005).

Although phytoestrogens may be extensively metabolized and biotransformed by the rumen microflora (Adams 1995), ruminal microorganisms may take, however, six to ten days to fully adapt to these substrates, so genistein may produce estrogenic effects in sheep during the first few days after introduction of the phytoestrogenic diet. Lundh and colleagues (1990) demonstrated that the total amounts of the plant estrogens and their metabolites in ovine blood plasma increased successively and reached the maximum level after about three hours of intake. Studies on rats showed that following intraperitoneal administration, genistein rapidly appears in brain and then in microdialysate fluid from the corpus striata together with its metabolite *p*-ethyl-phenol (Setchell 1998). Following prolonged oral administration or exposition *in utero* and throughout maternal milk, from dietary administration to the mothers, genistein predominantly concentrated in rat reproductive organs rather than in other peripheral tissues (Chang et al. 2000, Setchell 1998). The dose of genistein used in our experiment probably does not mimic the concentration of phytoestrogen, which occurs within the brain after ingestion

of oestrogenic feeds; however, its pharmacological effect on GH secretion in ewes is similar to that of 17 β -estradiol.

The regulation of GH secretion by estrogens is still being discussed and may involve both the pituitary and hypothalamic levels, emphasizing the most basic elements of this system, the somatotropes, GHRH, and SS (Chowen et al. 2004). Scanlan and Skinner (2002) showed that only a few GH-producing cells expressed ER and suggested that any direct modulatory effect of estrogens on the somatotropes, through the ER, could be minimal. In bovines, pituitary GH cells were unresponsive to estradiol (Hassan et al. 2001); however in the rat, the direct influence of estrogens on somatotropes is still controversial (Carlsson et al. 1987, Gonzalez-Parra et al. 1996, Simard et al. 2004). GHRH is produced in the hypothalamic neurons of the arcuate nucleus (ARC) that project to the external zone of the median eminence (Bluet-Pajot et al. 1998, Scanlan and Skinner 2002). Secretion of this neuropeptide into the hypophyseal portal system provides a specific stimulus for the release of GH from the pituitary. The clusters of cells producing SS are located in several hypothalamic sites. However, the neurons most directly involved in the inhibition of GH secretion are located in the periventricular nucleus (PeVN) (Bluet-Pajot et al. 1998). Some differences between species in the localization of ER have also been found in the form of estrogenic modulation of these two neuropeptides. Shirasu and others (1990) first demonstrated that a certain population of GHRH neurons are targets for estradiol and indicated that estradiol could act directly on certain hypothalamic GHRH neurons in female rats. In contrast, a few SS cells in hypothalamic PeVN showed nuclear labeling with [3H]estradiol (Shirasu et al. 1990). The presence of neurons co-expressing SS and ER was shown also in the hypothalamic ventromedial nucleus (VMN) in both male and female rats (Herbison 1994), considering SS as rather a putative mediator of reproductive function through this site. A more recent study confirmed that most GHRH neurons in the hypothalamic ARC have ER α , but not ER β , and only a few SS cells in the PeVN and ARC have ER α or ER β , suggesting that in rats GHRH neurons are direct target cells for estrogens (Kamegai et al. 2001, Shimizu et al. 2005). In case of sheep, Scanlan and Skinner (2002) reported that ovine GHRH neurons do not express ER α , but

many ER α -expressing cells surround them. Moreover, about a third of SS-producing neurons of the VMN express ER α , which is in contrast to the PeVN (Scanlan et al. 2003), which is thought to be a major source of SS in the hypophyseal portal system (Bluet-Pajot et al. 1998). However, Scanlan and coauthors (2003) showed that estradiol is able to simultaneously increase *c-fos* immunoreactivity in both GHRH and SS neurons of the GH/LH surge, proving that two of the neurotransmitter systems known to control GH secretion are activated in response to estrogen. Based on the above observations, the cited authors suggested that in ewes, activation of GHRH and SS neurons by estrogens was most likely mediated through an indirect interneuronal pathway (Scanlan et al. 2003). Nevertheless, taking into account our results (changes in plasma GH concentration and storage), suppression of the inhibitory tone could predominate in the estrogenic action of genistein. Further studies are needed to clarify this mechanism, including other identified GH-releasing peptides, i.e. ghrelin (Tannenbaum and Bowers 2001) and/or neurotransmitter systems (Iqbal et al. 2005).

CONCLUSION

We have shown that genistein, a plant-derived isoflavone, is an effective stimulator of GH secretion in ewes and may exert its effect at the level of the CNS.

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