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Immunohistochemical Localization of Ghrelin in Testicular Tissues of Holstein Bulls

Research Article

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ABSTRACT

Ghrelin, a 28-amino acid acylated peptide, was identified as the endogenous ligand for the growth hormone (GH) secretagogue receptor and its role in reproduction has recently been established. It is believed that the site of ghrelin expression in testes may indicate its role in local regulation. This study investigated the immunohistochemical (IHC) localization of ghrelin in Holstein bull testis using a monoclonal anti-ghrelin antibody as primary antibody and donkey anti-rabbit IgG horseradish peroxidase (HRP) polyclonal antibody as secondary antibody. Samples of testis were collected from three Holstein bulls aged of 1.5 to 2 years old and preserved in 10% formalin for posterior inclusion in paraffin. Histological sections with 5 micron in thickness were prepared for IHC. Immunoreactions were assessed for spermatogenic cells and leydig and sertoli cells. It is believed that the site of ghrelin expression in the spermatogenesis process, leydig and sertoli cells, may indicate its role in local regulations. This is one of the first studies to provide molecular evidence for the presence of ghrelin within testicular tissue cells of Holstein bulls.

KEY WORDS ghrelin, Holstein bulls, leydig cell, sertoli cell, testis.

INTRODUCTION

There are complex endocrine interactions in the male reproductive system, because various cell types contribute to produce spermatozoa, including the regulation of several inter and / or intra gonadal hormones and growth factors. The role of follicular stimulating hormone (FSH) and testosterone in spermatogenesis as a whole are widely known (Łukaszyk *et al*. 2009). Recent improvements in the knowledge of the neuroendocrine systems regulating different important body functions led to detection of a close relation among the systems governing somatic growth, energy balance and reproduction. Indeed, this link has been long expected on the basis of the well-known requirement of acceptable energy stores for proper pubertal development, growth and fertility of bull (Casanueva and Dieguez, 1999). However, the molecular signals responsible for this combined regulation have recently been identified. In this regard, leptin and ghrelin are probably the most studied neuroendocrine peptides (Barreiro and Tena-Sempere, 2004). Ghrelin is an acylated 28-amino acid peptide hormone predominantly secreted by endocrine cells of the stomach (Kojima *et al*. 1999). Several studies suggested that ghrelin is involved in the regulation of growth hormone (GH) release, energy balance, food intake and body weight (Wren *et al*. 2000). Its effects are mediated via a 7-transmembrane G protein-coupled receptor, the GH secretagogue receptor type 1a (GHSR-1a) (Miller *et al*. 2005). In addition to these biological effects, there are evidences for its peripheral actions, including in the regulation of reproduction. For example, it has been shown that exogenous ghrelin inhibits luteinizing hormone (LH) secretion both *in vivo* and *in vitro* in rats (Fernandez-Fernandez *et al*. 2004). Immunoreaction and gene expression for ghrelin and its functional receptor

have been found in the hypothalamic region of the rat, porcine and human, an area which is known to be important in the control of reproduction (Cowley *et al*. 2003; Wenlong *et al*. 2008). Ghrelin and its receptor growth hormone secretagogue receptor-1a (GHSR-1a) are expressed in both male and female reproductive organs (Garcia *et al*. 2007; Dupont *et al*. 2010). In the males ghrelin plays a crucial role in the regulation of the hypothalamic-pituitary gonadal axis and it also exerts direct regulatory effects at the testis, involving key aspects of testis physiology, such as steroidogenesis, leydig cells proliferation and tubular functions (Muccioli *et al*. 2011; Wenlong *et al*. 2008).

From the results of several studies, ghrelin has been localized in the testis of different species. Ghrelin immunoreactivity has been localized in interstitial leydig cells in rat (Tena-Sempere *et al*. 2002) and less intensely, also in sertoli cells of rats (Ishikawa *et al*. 2007; Lorenzi *et al*. 2009).

The GHSR-1a has been also found in rodent and human germ cells (leydig and sertoli cells) (Barreiro *et al*. 2003; Gaytan *et al*. 2003). Ghrelin and GHSR-1a were immunolocalised in the stomach (abomasum), anterior pituitary gland, testis, ovary, and hypothalamic and hindbrain regions of the brain of sheep (Miller *et al*. 2005). Ghrelin has not been localized in the reproductive tissues of Holstein bulls yet, even though expression has been thoroughly documented in other species. As a first step toward understanding the interactions between ghrelin and fertility in Holstein bulls, the current study focused on cellular location of ghrelin in the reproductive tissues of Holstein bulls.

MATERIALS AND METHODS

Samples of testicular tissues were obtained from three Holstein bulls, aged between 1.5 and 2 years old. The testes were excised immediately after slaughter and fixed in 10% formalin and paraffin-embedded. In this study, 4-5 serial sections for each sample were used with µm sections, mounted on slides coated with a suitable tissue adhesive; section were deparaffinized and rehydrated. A heat-treatment was performed for antigen retrieval. To break protein cross-links a citrate buffer (10 m*M*, pH=6) was used. Finally, indirect IHC technique was performed after heat treatment for antigen retrieval using citrate buffer (10 m*M*, pH=6) for break of protein cross-links. Peroxide block (5 min) was used to neutralize endogenous peroxides. Protein block (5 min) was used to block non-specific binding sites. For ghrelin detection a mouse monoclonal anti-ghrelin antibody was used as primary antibody and donkey anti-rabbit IgG (HRP) polyclonal antibody as secondary antibody (abcam). The sections were incubated overnight with the primary antibody diluted 1/500 in PBS, in a moist chamber at 4 ˚C. Thereafter, slides were incubated with post primary block (30 min), followed by incubation (20 min) with a secondary antibody diluted 1/300 in PBS and developed peroxides activity with (diaminobenzidine) DAB working solution (15 min). The sections were finally counterstained with hematoxylin and dried and mounted with cytology glue. In the control, normal rabbit serum at the same dilution as the primary antibody alone was applied instead of primary antibody.

RESULTS AND DISCUSSION

Evaluation of the pattern of cellular expression of ghrelin protein in Holstein bull testis using immunohistochemistry demonstrated that ghrelin peptide was located in the interstitial leydig, sertoli cells and germ cells. Thus, strong ghrelin immunostaining was observed in leydig cells, sertoli cells and germ cells of testicular tissue (Figure 1 a and b). No ghrelin signal was detected in control sections (Figure 1 c and d). In addition, the sheep testicular tissue was used as positive control (Figure 1 e). Extensive research efforts following identification of ghrelin, as the endogenous ligand for the GHS receptor (Kojima *et al*. 1999; Barriro *et al*. 2002) have pointed out the involvement of this newly discovered molecule not only in the physiological regulation of GH secretion but also in a variety of additional biological functions, including feeding and neuroendocrine control (Furuta *et al*. 2001). In this context, an unexpected reproductive facet of ghrelin has recently emerged. Intracerebroventricular administration of ghrelin has been shown to rapidly suppress pulsatile LH secretion (Furuta *et al*. 2001). It is noteworthy that previous evidence indicated that, as is the case for ghrelin, GH-releasing hormone (GHRH), a hypothalamic key factor in the control of GH secretion, is expressed in rat leydig cells under the positive control of LH (Ciampani *et al*. 1992). However, the effects of these signals on Leydig cell endocrine function appear to be opposite because GHRH enhanced LH-induced cAMP production and steroidogenesis in leydig cell cultures (Ciampani *et al*. 1992) whereas ghrelin significantly inhibited human (chorionic gonadotropin) hCG-stimulated testosterone secretion *in vitro* (Tena-Sempere *et al*. 2002). We have provided evidence for the expression of ghrelin in bull testis, which is comparable with studies in the literature dealing with localization and function of ghrelin in male and female reproductive systems of human beings and other animals. The results of the present study show that ghrelin is expressed in Holstein bull leydig, sertoli cells and in the course of spermatogenesis process. These observations may suggest that ghrelin may regulate spermatogenesis in an autocrine and / or paracrine manner, because there is evidence that ghrelin is able to modulate testicular key functions, such as seminiferous tubule gene expression, testosterone secretion and leydig cells proliferation (Dupont *et al*. 2010).

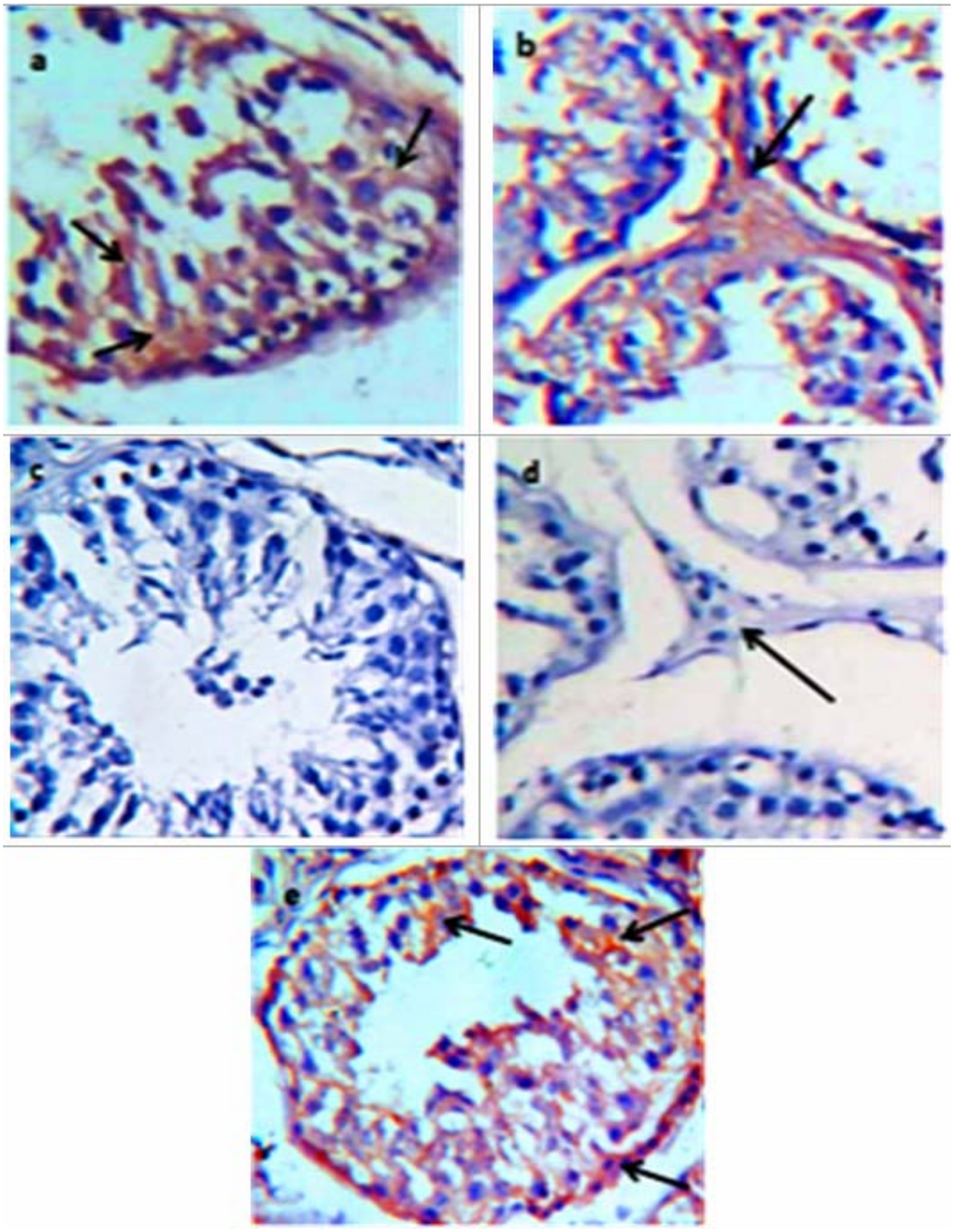

Figure 1 Immunohistochemistry detection of ghrelin in section of Holstein bull testis
Sections were incubated with mouse anti-ghrelin primary antibody and donkey anti rabbit IgG (HRP) polyclonal antibody as secondary antibody
Ghrelin expression was detected in the spermatogenesis process, sertoli cells (a) and leydig cells (b)
Arrows indicate positive staining
Panel c and d shows negative staining for ghrelin (negative control); positive control panel e) indicated immunoreactions in the spermatogenesis process and sertoli cells of sheep testicular tissue.
Scale bar= 50 µm

In addition, ghrelin was localised in leydig and sertoli cells of rats (Barreiro *et al*. 2003; Garcia *et al*. 2007; Dupont *et al*. 2010). Łukaszyk *et al*. (2012) indicated that GHSR-1a was expressed at the proacrosomal and acrosomal sites of rat spermatids and epididymal spermatozoa and provided evidence for its function in insemination.

CONCLUSION

This study has demonstrated for the first time that ghrelin is present in Holstein bull testis tissue, and more specifically in leydig cells, sertoli cells and germ cells. Therefore, the ghrelin ligand system may have a role (endocrine and/or paracrine) in the development (cellular proliferation) and function of the testicular tissues of the adult Holstein bull.

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