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.
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 mM, pH=6) was used. Finally, indirect IHC technique was performed after heat treatment for antigen retrieval using citrate buffer (10 mM, 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 1a and b). No ghrelin signal was detected in control sections (Figure 1c and d). In addition, the sheep testicular tissue was used as positive control (Figure 1e). 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. Intra-cerebroventricular 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). Lukaszyk 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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