Immunohistochemical, cellular localization and expression of inhibin hormone in the buffalo (*Bubalus bubalis*) adenohypophysis at different ages

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Abstract

The pituitary adenohypophysis was obtained from thirty buffaloes-cows, their age's ranges from one month to12 years. Sections of adenohypophysis tissues were immunostained for α, βa, and βb subunits of inhibin hormone. Positive immunostaining specific for the α subunits of inhibin were detected in the cells of the follicles of the adenohypophysis in all ages. Moreover, immunostaining specific for the inhibin βa subunits were strong positive at one month, weak positive at 4.5, 8 and 12 years and were negative at 8 months and 1.5 years. However, the immunostaining specific for the inhibin βb subunits were positive at one and 8 months, 8 and 12 years and weak positive at 4.5 years and negative at 1.5 year. RT-PCR analysis revealed that both α and βb subunits are expressed in all ages except at 1.5 years old animals while βa subunit is only expressed at young age.

Key words

Introduction

There is worldwide interest in buffalo as an animal for meeting the growing demands of meat and milk in developing countries. One of the major breeding problems in buffalos is its low reproductive efficiency. Ovarian cyclicity is regulated by hypothalamic hormones, gonadotro-

pins and ovarian steroids. Endocrine investigations of the hypothalamo-hypophysial-ovarian axis are needed to gain a better understanding of the reproduction physiology in buffalo and to be able to manipulate reproductive mechanisms to derive the maximum benefit from this animal (Mondal et al., 2007).

They regulate the reproductive system by acting on the pituitary gland and by blocking the synthesis of the FSH-β subunit and therefore the secretion of FSH (Burger and Igarashi, 1988; Attardi et al., 1992; Burger et al., 1998; Knight and Glister, 2001). Inhibin are heterodimeric molecules containing an α subunit and either a βa or βb subunit, which are connected to each other by a disulfide bond. If the dimer consists of a βa subunit the molecule is called inhibin A, and if it consists of a βb subunit the molecule is called inhibin B (Miyamoto et al., 1985;Robertson et al., 1985; Mason et al., 1986 and Burger and Igarashi, 1988). The three subunits are products of separate genes located on chromosomes 2 (α and βb subunit) and 7 (βa subunit) (Robertson et al., 2004). Inhibins are members of the transforming growth factor β (TGF-β) superfamily and are dimeric in structure (Evans and Groome, 2001; Robertson et al., 2004 a,b). Inhibin is a gonadal-derived heterodimeric glycoprotein hormone,

which selectively inhibits the secretion of follicle stimulating hormone (FSH) from the anterior pituitary gland (Burger, 1988 ; Kandiel et al., 2008). In addition to its action on FSH through hypothalamo–hypophyseal– ovarian axis, inhibin may also plays an important role in regulation of folliculogenesis through autocrine and paracrine control (Findlay, 1993).

Both α- and β-subunits of inhibin are generated by proteolytic cleavage of two independently synthesized large precursor molecules (Mason et al., 1985, 1986; Forage et al., 1986). In several species, the most predominant form of biologically active inhibin identified has a molecular weight (MW) of approximately 31–32 kDa (Robertson et al., 1985, 1986; Rivier et al., 1985; Fukuda et al., 1986; Miyamoto et al., 1985; Ling et al., 1985). In addition, inhibin of different MW has been isolated from various species including cattle (Fukuda et al., 1986; Sugino et al., 1992). Two major forms of inhibin (129 and 63 kDa) with little proteolytic cleavage/processing of the large precursor in the buffalo follicular fluid were determined by (Ganguly et al., 2010). The objectives of this study were to immunolocalize inhibin α and □ (βa and βb) subunits and to investigate the expression of inhibin mRNA in the buffalo-cows adenohypophysis.

Material and Methods

Animals and tissues

Thirty buffaloes-cows (*Bubalus bubalis*) pituitaries were collected from Al-Amar abattoir in kalyobia governorates, Egypt. Their ages were 1, 8, 18 months 4.5, 8 and 12 years , five animals each. Some animals of the old ages 12 years groups showed silent heat. The samples were preserved in Bouin's fluids, dehydrated in ascending grades of ethanol, cleared in xylene and cut at 3μm for further processing.

Materials

Rabbit polyclonal antibody against (Tyro30) porcine inhibin α chain (1–30) NH2, rabbit anti-cyclic inhibin βA (81–113)-NH2 (Code 334-274-ET) and rabbit anti-cyclic inhibin βB (80–112)-NH2 (Code 305-25-D) were purchased from WAKO Pharmaceutical Company, Tokyo.

Immunohistochemical localization

Immunohistochemical staining was generally performed as described by (Mousa and Mousa, 1999). Formalin-fixed and paraffin-embed-ded tissue sections were stained immunohistochemically with antibodies against the alpha and beta-subunits of inhibin. Sections were deparaffinized in xylene, rehydrated through graded ethanol, and washed in phosphate-buffered saline (PBS; pH 7.4) for two times 10 min each. The sections were then incubated with 0, 25% (v/v) biotinylated secondary antibody (Vector Laboratories) diluted in PBS containing 10% normal goat serum to reduce the non specific binding. The sections were then incubated with 2% avidin-biotin complex. The reaction were visualized with di-amino benzidine tetrahydrochloride (DAB) (Sigma) 0.025% N 0.05 M PBS. The stained sections were counterstained with Mayer's haematoxyline (Bancroft et al., 1996). After that, the sections were washed in tap water, dehydrated in alcohol, cleared in xylene, and mounted in DPX. Photographed by Leica digital microscopy. Control was performed by exposing section to normal goat serum instead of primary antibodies.

RT-PCR analysis

All primers were manufactured by BIORON GmbH-Germany. It were designed to have a melting temperature of 60 °C using the DNASTAR Lasergene version 4.0 expert sequence analysis software and compared with the Genbank using the program BLAST available at www.ncbi.nlm.nih.gov.

Total RNA was extracted from 20 mg of the pituitary gland using QIAGEN RNeasy Mini Kit (50) (Ger-

many) and tested on denatured agarose gel by electrophoresis. Two micrograms of the total RNA were reverse transcribed into cDNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, USA). The reactions were incubated for 60 min at 42oC and terminated by heating at $70^{o}C$ for 5 min. 2 µl of the cDNA was used for PCR that was carried out in the Perkin Elmer 9600 using a reaction mix containing 1 unit of Ampli Taq Gold (PE Applied Biosystems), 1x buffer, 1.5 mM MgCl2, 2.5 mM dNTPs and 20 pmol of each forward and reverse primers for 18S-rDNA (positive control), inhibin α, βa or βb primers (table 1). The total volume was 50 µl. An initial cycle of 10 min at $95^{o}C$, 45 seconds at $55^{o}C$ and 1 minute at $72^{o}C$ was performed. PCR conditions included initial heating at $95^{o}C$ for 5 min then annealing at $55^{o}C$ for 45 sec and extension at $72^{o}C$ for 1 min for 35 cycle, followed by final extension at $72^{o}C$ for 7 minutes. A negative control containing RNA instead of c DNA was used to rule out genomic DNA contamination. The PCR products were confirmed by 2% agarose gel electrophoresis.

A 100 bp DNA ladder molecular weight marker (invitrogen, USA) was used to confirm expected molecular weight of the amplification product (Tabel, 1).

Results

Positive immunostaining specific for the α, βa and βb subunits of inhibin were observed in the basophilic cells (Gonadotrophs) of the buffalo-cows adenohypophysis at one month of age. (Plate.1). Positive immunostaining specific for the α and βb subunits and negative immunostaining specific for βa were observed in the basophilic cells (Gonadotrophs) of the buffalo-cows adenohypophysis at 8 months. (Plate.2). Positive immunostaining specific for the α subunits and negative immunostaining specific for the βa and βb subunits in the basophilic cells (Gonadotrophs) of the buffalo-cows adenohypophysis at 1.5 years old age. (Plate.3). Positive immunostaining specific for the α subunits and weak positive immunostaining specific for the βa and βb subunit in the basophilic cells (Gonadotrophs) of the buffalo-cows adenohypophysis at 4.5 years. (Plate. 4).

Positive immunostaining specific for the α and βb subunits and weak positive immunostaining specific for the βa subunit in the basophilic cells (Gonadotrophs) of the buffalo-cows adenohypophysis at 8 and 12 years were observed. (Plate.5 and 6 respectively).

Expression of inhibin α and βb subunits mRNA were detected in the adenohypophysis of the Egyptian water buffalo *Bubalus bubalis* at all ages studied relative to internal standard (563 bp) of 18S r DNA. Inhibin α band with molecular weight of 217 bp was detected in all animal ages as shown in figure (7) whereas inhibin βa band (209 bp) was detected only in the early age (1 month). Expression of inhibin βa mRNA was not detected in other ages. The inhibin βb band with molecular weight of 218 bp was detected in all ages except at 1.5 years.

Discussion

Buffalo has recently gained a lot of attention due to its high milk yield with high fat percentage, tolerance to hot and humid climate, lean meat, draught ability and a reasonable growth rate on roughage feeding. Buffalo, as domestic animal with its predominant home tract in South East Asia and Mediterranean, has found great acceptance in the rural economies of Asia and Africa. Some peculiar problems of this species as late maturity, silent heat coupled with poor expression of oestrus, irregular oestrous cyclicity, seasonality in breeding, anoestrus, low conception rate, long postpartum interval and repeat breeding etc. have been the major constraints leading

to low productivity (Madan et al., 1993; 1996). A considerable attention has been focused in the last two decades on utilizing reproductive endocrinology as a means to identify problems specific to this species and to devise means for augmenting its reproductive performance.

Activin and inhibin were first purified from mammalian follicular fluid as factors that regulate the secretion of follicle-stimulating hormone (FSH) from pituitary cells in culture (Ling et al., 1985, 1986 a,b; Mason et al.,1985; Rivier et al., 1985; Vale et al., 1986). The inhibin is also secreted from extragonadal source such as the pituitary gland and brain (Lau and Li, 1987; Roberts et al., 1989; Schlatt et al., 1991), the placenta (McLachlan et al., 1986), the adrenal , pituitary glands , lung and the spleen (Meunier et al.,1988). It is now clear that activins and their two functional modulators, inhibins and follistatins, control reproductive function by exerting effects on the pituitary as well as throughout the reproductive axis. The endocrine function of inhibins as gonadal feedback modulators of FSH production appears to be more physiologically relevant (De Kretser et al., 2002; Welt et al., 2002 ; Bilezikjian et al.,2004). Expression of the inhibin subunits, in particular the β-subunits A and B, is widespread, and activins

act as autocrine/paracrine regulators of cell functions in a wide range of tissues, including the gonads, adrenal gland, liver, bone and bone marrow (Vale et al., 1990; Spencer et al., 1992; Centrella et al.,1994; Zhang et al., 1996; De Kretser et al., 2000).

Inhibin is a heterodimeric peptide hormone produced in the ovary that antagonizes activin signaling and FSH synthesis in the pituitary. The inhibin β-subunit interacts with the activin type II receptor (ActRII) to functionally antagonize activin. The inhibin α-subunit mature domain (N-terminus) arose relatively early during the evolution of the hormone, and inhibin function is decreased by an antibody directed against the α-subunit N-terminal extension region or by deletion of the N-terminal region. The α-subunit N-terminal extension region interacts with the activin type I receptor (ALK4) to antagonize activin signaling in the pituitary. (Jie Zhu et al.,2012). The passive immunization of mares against inhibin during the follicular phase of the estrous cycle results in a marked increase in plasma concentrations of FSH and estradiol-17f3, and in the ovarian follicle population. These data indicate that immune-neutralization of endogenous inhibin enhances a rapid growth of a large number of follicles and results in multiple ovulations in mares,

largely due to increasing secretion of FSH from the pituitary gland. The control of secretion of FSH by a blockade of inhibin bioactivity may become a new method for controlling folliculogenesis and ovulation rate in mares**.** (Nambo et al., 1998).

Both inhibin βa and βb subunits were detected in the pituitary gland of buffalo-cows by immunocytochemistry. The immunoreactivity of inhibin βb was stronger than that of the inhibin βa subunit. The immunoreactivity for inhibin βb appears to be localized in close contact with gonadotrophs. (Bilezikjian et al., 2004) , these results augmented our findings, that βb was stronger in reaction than that of the βa. The inhibin-α subunits were positive immunostaining in the adenohypophysis cells (Gonadotrophs) at 1, 8,18 months. These results were supported by Terzano (2010) who stated that, plasma Inhibin-A concentrations began to gradually increase four weeks before the onset of puberty (18,83 and 13,07 ng/ml, respectively at puberty) and this increase continued throughout the peripuberal period . So, these results imply that inhibin-A can be used as a novel marker of the onset of puberty in buffalo heifers.

In the reproductive system, activins and inhibins have been found to regulate the release of gonadotro-

phinreleasing hormone (GnRH) from the hypothalamus (Calogero et al., 1998; Peng and Mukai, 2000) and gonadotrophins (GTHs) from the pituitary (Bilezikjian et al., 2001 and Uchiyama et al., 2000).

Inhibin, a suppressor of FSH secretion, is composed of an a chain covalently linked to a β chain; whereas activin, a stimulator of FSH secretion, is composed of a dimer of the βchain. Since there are two structurally related βchains, inhibin has two isoforms (aβA as inhibin A, and aβB as inhibin B), and activin has three isoforms (βAβA as activin A, βAβB as activin AB, and βBβB as activin B). Expression of both mRNAs for inhibin a and βB chains has been demonstrated in the rat pituitary (Meunier et al., 1988; Dalkin et al., 1998).

The importance of inhibins in the control of the reproductive function has been reported (De Kretser et al., 2002; Medan et al., 2007) and it appears that βb-subunit might be produced in small developing follicles, which is replaced by the inhibin βa-subunit as the follicles approach the preovulatory stage (Medan et al., 2007). In cattle (Kaneko et al., 2002) and goats (Medan et al., 2005) an inverse relationship between FSH and inhibin-A was demonstrated, suggesting the key role of inhibin-A produced by domi-

nant follicle(s) in terminating the transient peaks of FSH secretion (Medan et al., 2007).

Inhibin is involved in folliculogenesis by negative feedback effects on FSH secretion in buffalo (Mondal et al., 2000b). Peripheral ir-inhibin concentrations were negatively correlated with peripheral progesterone concentrations in buffaloes indicating that corpus luteum is not a source of peripheral inhibin in buffaloes (Mondal et al., 2000a; Mondal et al., 2003a). The peripheral plasma ir-inhibin concentrations were compared between buffaloes which exhibited over oestrus and those which had silent estrus reported that plasma ir-inhibin levels were significantly higher in buffaloes that exhibited silent oestrus indicating that this could be one of the reasons responsible for poor expression of oestrus (Mondal et al., 2002a, b; 2003b).

Inhibin-A may provide an index of follicular development (Lockwood et al., 1996), but studies exploring the relationship between controlled ovarian stimulation, inhibin levels and ovarian follicular development in ruminants are limited (Gonzalez-Bulnes et al., 2002;Gonzalez-Bulnes et al., 2004).

A significant increase in inhibin levels through late luteal to perioestrus

phase is suggestive of a vital role of inhibin in folliculogenesis (Mondal et al., 2000a; 2001b).

When choosing a semi-quantitative protocol to determine RNA expression levels by RT-PCR, many parameters must be taken into consideration (Freeman et al., 1999). One of these parameters is necessity to study different markers in the same sample. An oligo (dT)18 primer was used instead of gene specific primers in the first strand cDNA formation to synthesize cDNA from the total RNA present in the samples, which will include a common expressed genes (housekeeping genes) like 18S rRNA (Zhou et al., 2010), as well as the investigated target genes (inhibin α, βa, and βb). This is a very convenient method when a single RNA marker has to be analyzed in a large number of samples. On the other hand, assaying several RNAs on the same sample, we preferred to work on the same cDNA product in order to reduce the variability due to the reverse transcription reaction.

Popovics et al. (2011) examined the regulation of gene expression of α, βb, and follistatin by local and endocrine hormones (GnRH) in pituitaries from female rats and in perifused pituitary cells by RT-PCR.

The expression of inhibin βb was higher than βa in the pituitary gland cells, these result was augmented by (Feng et al., 1995; Tena-Sempere et al., 1999; Toebosch et al., 1988), which stated the Sertoli cell produces two βb subunit mRNA transcripts, which are under differential regulation, and normal expression levels of the βb -subunits appear to be considerably higher than those of the βa-subunit. inhibin βa is actually the predominant form (MacConeilly et al., 2002).

References

Attardi B, Vaughan J, Vale W (1992): Regulation of FSH beta messenger ribonucleic acid levels in the rat by endogenous inhibin. Endocrinology;130: 557.

Bancroft JD, Stevens A, Turner DR (1996): Theory and practice of histological techniques. 4th ed. London/Toronto: Churchill Livingstone;

Bilezikjian LM, Blount AL, Corrigan AZ, Leal A, Chen Y, Vale WW (2001): Actions of activins, inhibins, and follistatins: implications in anterior pituitary function. Clin. Exp. Pharmacol. Physiol; 28 (3): 244–248.

Bilezikjian Louise M , Amy L Blount, Angela MO Leal, Cindy J Donaldson, Wolfgang H

Fischer , Wylie W Vale (2004): Autocrine/paracrine regulation of pituitary function by activin, inhibin and follistatin. Molecular and Cellular Endocrinology;225 :29–36.

Burger HG , Igarashi M(1988): Inhibin: definition and nomenclature including related substances. Journal of Clinical Endocrinology and Metabolism .; 66: 885–886.

Burger HG (1988): Inhibin: definition and nomenclature, including related substances. Journal of Endocrinology; 117: 159–160.

Burger HG, Groome NP, Robertson DM (1998): Both inhibin A and B respond to exogenous follicle-stimulating hormone in the follicular phase of the human menstrual cycle. J. Clin. Endocrinol. Metab;83: 4167.

Calogero AE, Burrello N, Ossino AM, Polosa PD,_Agata R (1998): Activin-A stimulates hypothalamic gonadotropin-releasing hormone release by the explanted male rat hypothalamus: interaction with inhibin and androgens. J. Endocrinol;156: 269–274.

Centrella M, Horowitz MC, Wozney JM, McCarthy TL(1994): Transforming growth factor-beta gene family members and bone. Endocr. Rev;15: 27–39.

Dalkin A C, Haisenleder D J, Gilrain J T, Aylor K, Yasin M, Marshall J. C (1998): Regulation of pituitary follistatin and inhibin/activin subunit messenger ribonucleic acids (mRNAs) in male and female rats: Evidence for inhibin regulation of follistatin mRNAin females. Endocrinology;139: 2818–2823.

De Kretser DM, Hedger MP, Loveland KL, Phillips DJ (2002): Inhibins, activins and follistatin in reproduction. Hum. Reprod. Update;8: 529–541.

De Kretser DM, Meinhardt A, Meehan T, Phillips DJ, O'Bryan MK, Loveland KA (2000): The roles of inhibin and related peptides in gonadal function. Mol. Cell. Endocrinol ;161: 43–46.

Evans LW, Groome NP (2001): Development of immunoassays for inhibin, activin and follistatin. In: Muttukrishna S, Ledger W (Eds.) Inhibin, Activin and Follistatin in Human Reproductive Physiology. Imperial College Press, London; p: 11.

Feng ZM, Wu AZ, Chen CL(1995): Characterization and regulation of two testicular inhibin/activin bB-subunit messenger ribonucleic acids that are transcribed from alternate

initiation sites. Endocrinology; 136: 947–955.

Findlay JK (1993):An update on the roles of inhibin, activin and follistatin as local regulators of folliculogenesis. Biology of Reproduction; 48: 15–23.

Forage RG, Ring JM, Brown RW, Mclnerney BV, Cobon GS, Gregson, RP,Robertson DM, Morgan FJ, Hearn MTW, Findlay JK, Wettenhall REH, Burger HG, de Kretser DM (1986): Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. Proceeding of the National Academy of Sciences, USA; 83: 3091–3095.

Freeman WM, Walker SJ , Vrana KE (1999): Quantitative RT-PCR: pitfalls and potential. BioTechniques;26: 112-125

Fukuda M,Miyamoto K, Hasegawa Y, Nomura M, Igarashi M, Kangawa K, Matsuo H (1986): Isolation of bovine follicular fluid inhibin of about 32-kDa.Molecular and Cellular Endocrinology;44: 55–60.

Ganguly A , Indrajit Ganguly , Sanat K Meur (2010): Multiple molecular forms of inhibin in buffalo (Bubalus bubalis) ovarian follicular fluid. Research in Veterinary Science; 89:14–19

Gonzales-Bulnes A, Santiago-Moreno J, Garcia-Garcia RM, Souza CJH, Lopez-Sebastian A, Mc Neilly AS (2004): Effect of GnRH antagonists on gonadotrophin secretion, follicular development and inhibin A secretion in goats. Theriogenology; 61(5):977-985.

Gonzales-Bulnes A ,Santiago-Moreno J, Cocero MJ, Souza CJH, Groome NP, Garcia-Garcia RM, Lopez-Sebastian A , Baird TD (2002): Measurement of inhibin A and follicular status predict the response of ewes to superovulatory FSH treatments. Theriogenology;57:1263-1272.

Jie Zhu S Jack Lin, Chao Zou, Yogeshwar Makanji, Theodore S Jardetzky, Teresa K Woodruff (2012): Inhibin α-Subunit N-terminus Interacts With Activin Type IB receptor To Disrupt Activin Signaling. The American Society for Biochemistry and Molecular Biology; In press

Kandiel MMM, Watanabe G, Li JY, Manabe N, El-azab AI , Taya K(2008): Physiological roles in regulation of FSH secretion and follicular development during early pregnany in goats. Dom Anim Endocrinology;35:157-163.

Kaneko H, Noguchi J, Kikuchi K, Todoroki J, Hasegawa Y (2002):Alterations in peripheral concentrations of inhibin A in cattle studied using a time-resolved immunofluorometric assay; Relationship with estradiol and folliclestimulating hormone in various reproductive conditions. Biology of Reproduction; 67:38–45.

Knight PG, Glister C (2001): Potential local regulatory functions of inhibins, activins and follistatin in the ovary. Reproduction;121: 503.

Lau YL, Li C (1987): Localization of cu-inhibin-31 in rodent testis and brain by immunocytochemical procedure and Western blot analysis. Biochem. Biophys. Res. Commun;145: 81-89.

Ling N, Ying SY, Ueno N, Esch F, Denoroy L,Guillemin R (1985):Isolation and partial characterization of aMr 32,000 protein with inhibin activity from porcine follicular fluid. Proc. Natl. Acad.Sci. USA;82: 7217–7221.

Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin R(1986a): Pituitary FSH is released by a heterodimer of the beta-subunits from the two forms of inhibin. Nature;321:779–782.

Ling Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin R(1986b): A homodimer of the beta-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. Biochem. Biophys. Res. Commun;138: 1129–1137.

Lockwood G M, Muttukrishna S, Groome N P, Knight P G, Ledger W L (1996):Circulating inhibins and activin A during GnRH-analogue down-regulation and ovarian hyperstimulation with recombinant FSH for in-vitro fertilization- embryo transfer. Clinical Endocrinology;45(6):741–748.

MacConell LA, Leal AM, Vale WW(2002): The distribution of beta glycan protein and mRNA in rat brain, pituitary, and gonads: implications for a role for betaglycan in inhibin-mediated reproductive functions. Endocrinology;143: 1066–1075.

Madan M L, Das S K, Palta P (1996):Application of reproductive technology to buffaloes. Anim. Reprod. Sci ; 42 : 299-306.

Madan M L, Prakash B S, Jailkhani S, Singla S K, Palta P , Manik R S (1993): Buffalo endocrinology with special reference to embryo transfer. Embryo

Biotechnology Centre, NDRI, Karnal, India; pp :32.

Mason A J, Hayflick J S, Ling N, Esch F, Ueno N, Ying SY,Guillemin R, Niall H, Seeburg P H(1985): Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-beta. Nature; 318: 659–663.

Mason AJ, Niall HD, Seeburg PH (1986): Structure of two human ovarian inhibins. Biochem. Biophys. Res. Commun;135: 957.

McLachlan RI, Healy DL, Robertson DM, Burger HG, de Kretser DM (1986):The human placenta: a novel source of inhibin. Biochem. Biophys. Res. Commun;140: 485-490.

Medan MS, Arai KY, Watanabe G, Taya K (2007): Inhibin: Regulation of reproductive function and practical use in females. Animal Science Journal; 78:16–27.

Medan MS, Watanabe G, Sasaki K, Groome NP, Sharawy S, Taya K (2005): Follicular and hormonal dynamics during the estrous cycle in goats. Journal of Reproduction and Development;51: 455–463.

Meunier H, Rivier C, Evans R M, Vale W (1988): Gonadal and extragonadal expression of inhibin a, bA and bB subunits in various tissues predicts diverse functions. Proc. Natl. Acad. Sci. USA;85: 247–251.

Miyamoto K, Hasegawa Y, Fukuda M, Nomura M, Igarashi M, Kangawa K, Matsuo H (1985): Isolation of porcine follicular fluid inhibin of 32KDaltons. Biochem. Biophys. Res. Commun;129: 396.

Mondal S, Prakash B S, Palta P(2000b): Relationship between peripheral plasma inhibin and FSH in cycling Murrah buffaloes (Bubalus bubalis). J. Endocrinol. (Supp.); 167:67 (Abstr.)

Mondal S, Prakash B S, Palta P (2001b): Comparison of changes in peripheral plasma inhibin in relation to oestrous cycle between cows and buffaloes. In: Proceed. Br. Soc. Anim. Sci., London, UK; p: 221.

Mondal S, Prakash B S, Palta P(2003a): Relationship between peripheral plasma inhibin and progesterone concentrations in Sahiwal cattle (Bos indicus) and Murrah buffaloes (Bubalus bubalis). Asian-Aust. J. Anim. Sci; 16 (1): 6-10.

Mondal S, Prakash B S, Palta P(2003b): Peripheral plasma

inhibin concentrations in relation to expression of oestrus in Murrah buffaloes (Bubalus bubalis). Ind. J. Anim. Sci ;73 (4):405-407.

Mondal S, Palta P, Prakash B S(2000a): Peripheral inhibin concentrations in relation to stage of oestrous cycle and progesterone in Sahiwal cows and Murrah buffaloes. In: Proceedings of 14th International Congress on Animal Reproduction, Stockholm, Sweden; 1:209.

Mondal S, Palta P, Prakash B S (2002a): Peripheral plasma inhibin concentrations in relation to overt and silent oestrus in Sahiwal cows and Murrah buffaloes. Biol. Reprod; (Supp 1) 66:261 (Abstr.).

Mondal S, Palta P , Prakash B S (2002b): Peripheral plasma inhibin concentration in relation to oestrus expression in Murrah buffaloes. In: Proceedings of 89th Session of Indian Science Congress Association, Lucknow, India; p: 64-65.

Mondal S , Prakash B S, Palta P(2007): Endocrine Aspects of Oestrous Cycle in Buffaloes (Bubalus bubalis): An Overview. Asian-Aust. J. Anim. Sci ;Vol. 20, No. 1 : 124 – 131.

Mousa MA, Mousa SA (1999): Immunocytochemical study on the localization and distribution of the somatolactin cells in the pituitary gland and the brain of Oreochromis niloticus (Teleostei, Cichlidae). Gen. Comp. Endocrinol.;113: 197– 211.

Nambo Y, Kaneko H, Nagata S, Oikawa M T Yoshihara, Nagamine N, Watanabe   G K (1998): Effect of passive immunization against inhibin on FSH secretion, folliculogenesis and ovulation rate during the follicular phase of the estrus cycle in mares. Theriogenology.; 50:545-557.

Peng C, Mukai ST (2000): Activins and their receptors in female reproduction. Biochem. Cell Biol.;78 (3): 261–279.

Popovics P, Rekasi Z, Stewart AJ, Kovacs M (2011): Regulation of pituitary inhibin/activin subunits and follistain gene expression by GnRH in female rats. J. Endocrinol;210(1): 71-9 Apr. 13.

Rivier J, Spiess J, McClintock R, Vaughan J, Vale W (1985): Purification and partial characterization of inhibin from porcine follicular fluid. Biochem. Biophys. Res. Commun;133: 120–127.

Roberts V, Meunier H, Vaughan J, Rivier J, Rivier C, Vale W, Cawchenko P (1989): Produc-

tion and regulation of inhibin subunits in pituitary gonado- tropes. Endocrinology;124: 552-554.

Robertson DM, Burger HG, Fuller PJ (2004a): Inhibin/activin and ovarian cancer. Endocr. Relat. Cancer ;11: 35.

Robertson DM, de Vos FL, Foulds LM, McLachlan RI, Burger HG, Morgan FJ, Hearn MTW, de Kretser DM (1986): Isola- tion of a 31 kDa form of inhibin from bovine follicular fluid. Mo- lecular and Cellular Endocri- nology;44: 271–277.

Robertson DM, Foulds LM, Lever- sha L,Morgan FJ, Hearn MT, Burger HG, Wettenhall RE, de Kretser D M (1985):Isolation of inhibin from bovine follicular fluid. Biochem. Biophys. Res. Commun;126: 220.

Robertson DM, Pruysers E, Burger HG, Jobling T, McNeilage J, Healy D(2004b): Inhibins and ovarian cancer. Mol. Cell. En- docrinol ;225: 65.

Schlatt S, Weinbauer GF, Nieschag E (1991): Inhibin-like gonado- trophin-like immunoreactivity in pituitary cells of male mon- keys, Macaca fascicularis, Macaca mulatta. Cel1 Tissue Res.; 265: 203-209.

Spencer SJ, Rabinovici J, Mesiano S, Goldsmith PC, Jaffe RB(1992): Activin and inhibin

in the human adrenal gland. Regulation and differential ef- fects in fetal and adult cells. J. Clin. Investig;90: 142–149.

Sugino K, Naka Mura T, Takio K, Miya Moto K, Hasegawa Y, Igarashi M, Titani K, Sugino H (1992): Purification and char- acterization of high molecular weight forms of inhibin from bovine follicular fluid. Endocri- nology;130: 789–796.

Tena-Sempere M, Kero J, Rannikko A, Yan W, Huhtaniemi I(1999): The pattern of inhibin/activin a- and bB-subunit messenger ribonucleic acid expression in rat testis after selective Leydig cell destruction by ethylene dimethane sulfonate. Endocri- nology;140: 5761–5770.

Terzano GM (2010): Inhibin A: ex- perience and role in buffalo reproductive physiology.Rev. Vet. 21, Sup 1: 172-178.SAS, 2006. The SAS System for Windows, Release 9.2. SAS Institute Inc., Cary, NC, USA

Toebosch AM, Robertson DM, Trapman J, Klaassen P, de Paus RA, de Jong FH, Grootegoed JA(1988): Effects of FSH and IGF-I on immature rat Sertoli cells: inhibin a- and b-subunit mRNA levels and inhibin secretion. Mol. Cell. Endocrinol;55: 101–105.

Uchiyama H, Koda A, Komazaki S, Oyama M, Kikuyama S(2000): Occurrence of immunoreactive avtivin/inhibin beta(B) in thyrotrophs and gonadotrops in the bullfrog pituitary: possible paracrine/autocrine effects of activin B on gonadotropin secretion. Gen. Comp. Endocrinol;118: 68–76.

Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J. (1986):Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature;321: 776–779.

Vale WW, Hseuh A, Rivier, C, Yu J (1990): The inhibin/activin family of hormones and growth factors. In: Sporn, M.A., Roberts, A.B. (Eds.), Peptide growth factors and their receptors. Handbook of Experimental Pharmacology; vol 95. Springer, Berlin, pp: 211–248.

Welt C, Sidis Y, Keutmann H, Schneyer A (2002): Activins, inhibins, and follistatins: from endocrinology to signaling: a paradigm for the new millennium. Exp. Biol. Med Maywood;227: 724– 752.

Zhang YQ, Kanzaki M, Mashima H, Mine T, Kojima I(1996): Characterization of the activin receptor in cultured rat hepatocytes. Hepatology.; 24: 446–450.

Zhou RX, Meng T, Meng, H B , Cheng DX (2010):Selection of reference gene in transcription analysis of gene expression of the mandarin fish, Siniperca chuasti. Zoological Research ; 31(2): 141-146.

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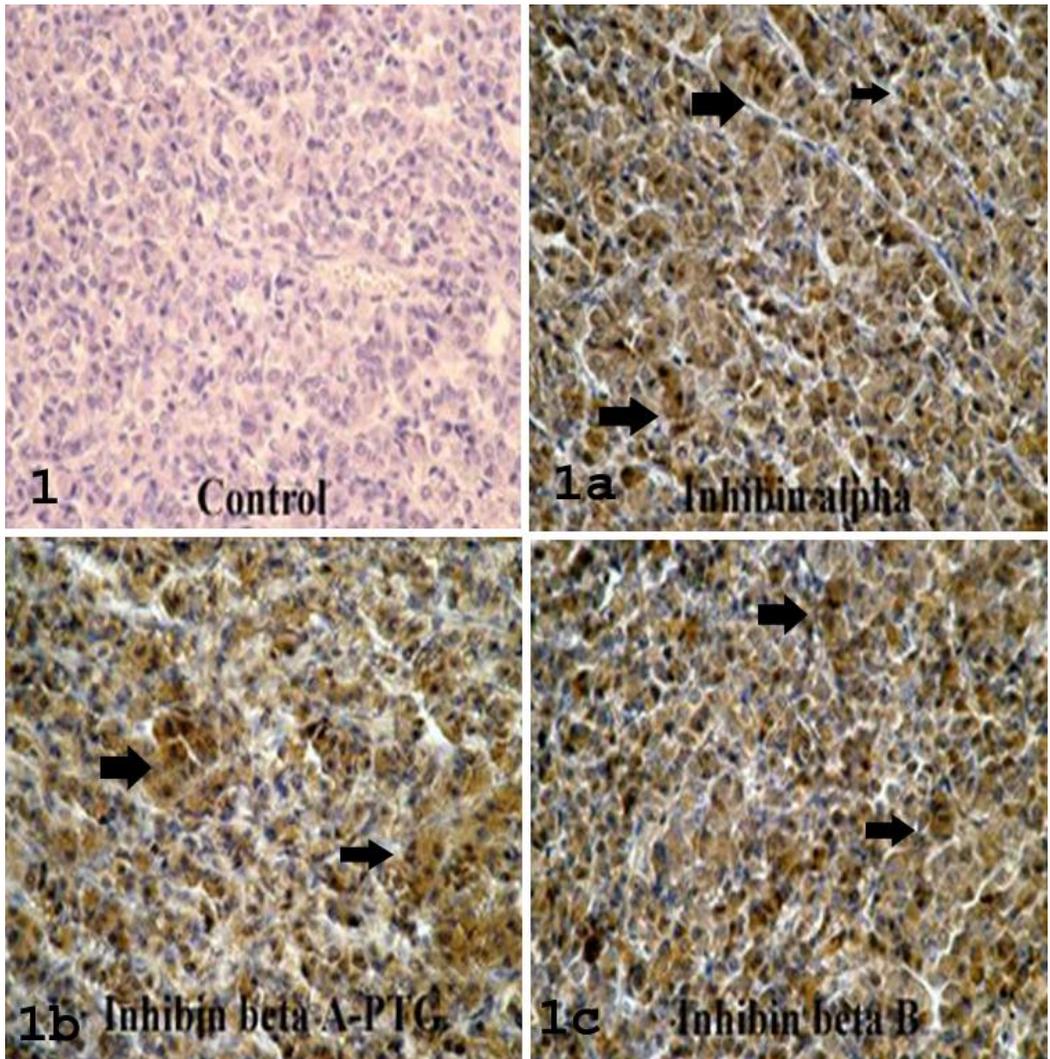
Plate (1): Photomicrograph of the buffalo-cows adenohypophysis at 1 month showing, control immunostaining (1), positive immunostaining specific for the inhibin α (1a) arrows, βa (1b) and βb (1c) subunits (arrows). X100
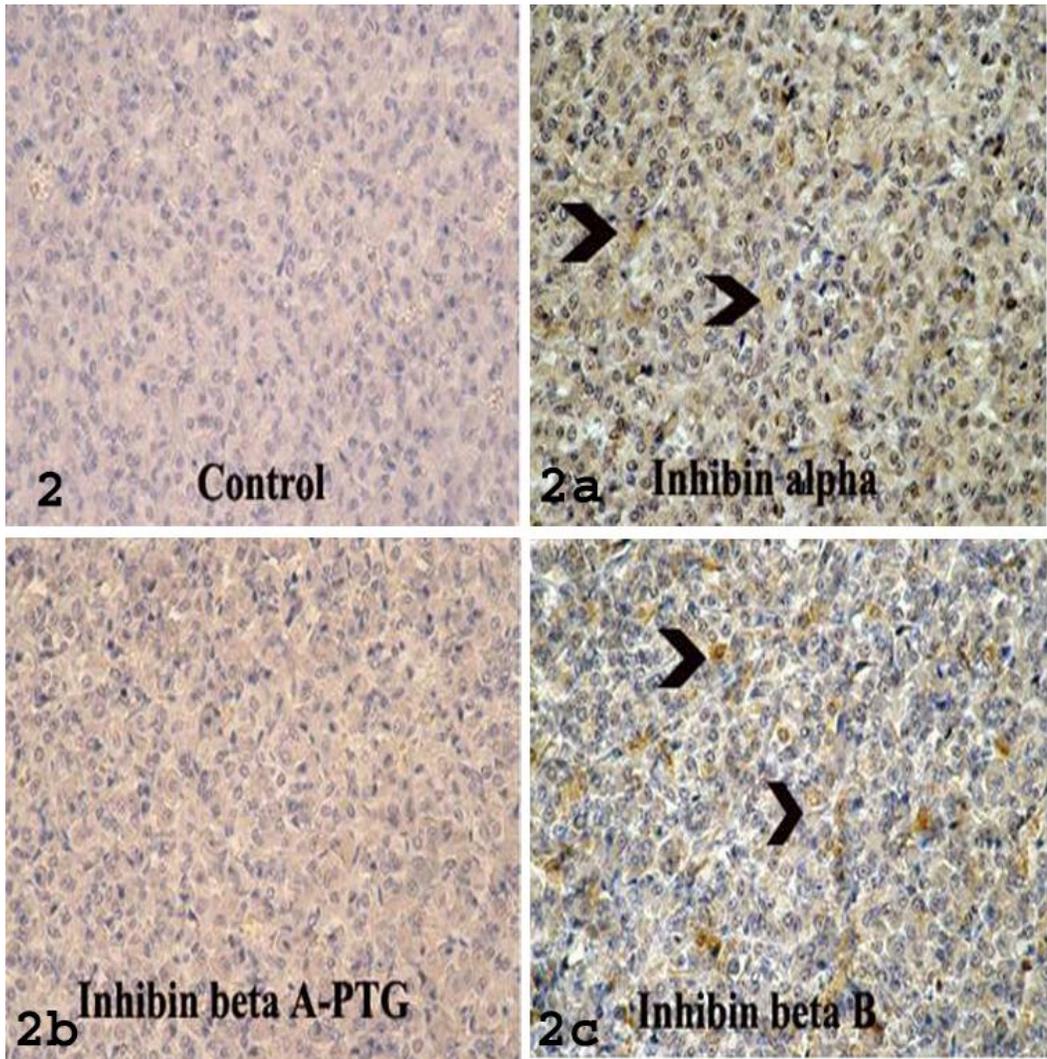
Plate (2): Photomicrograph of the buffalo-cows adenohypophysis at 8 months showing, control immunostaining (2), positive immunostaining specific for the inhibin α (2a), βb (2c) and negative immunostaining βa (2b) subunits (arrow heads) . X100
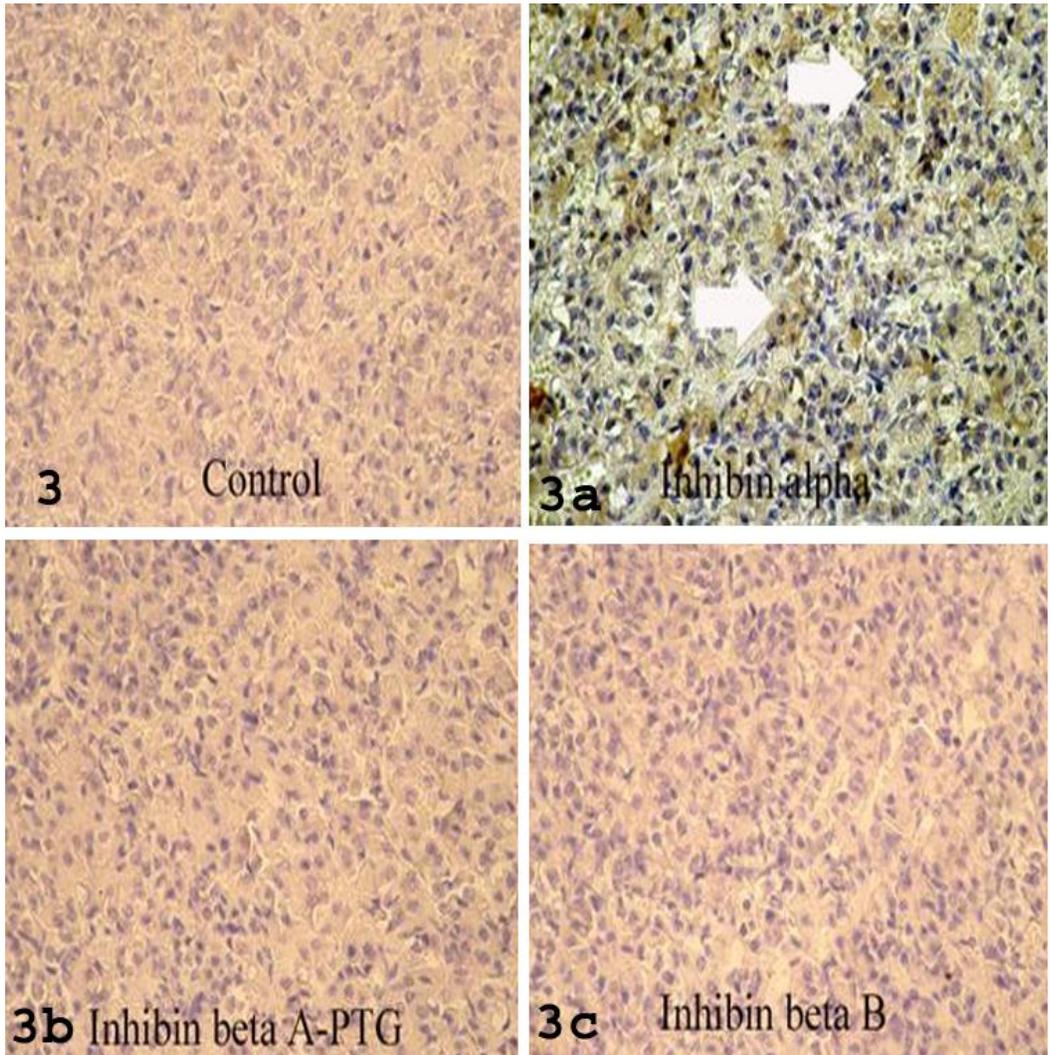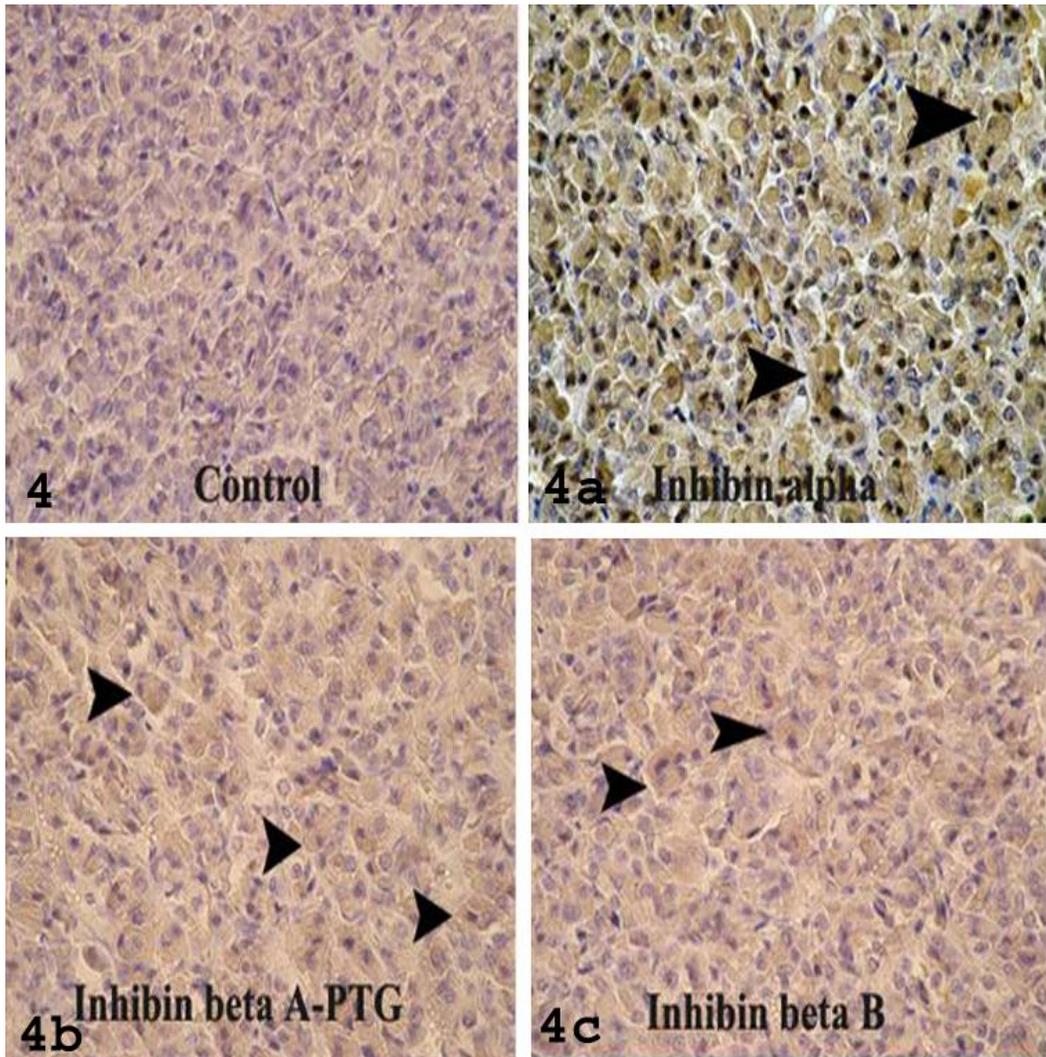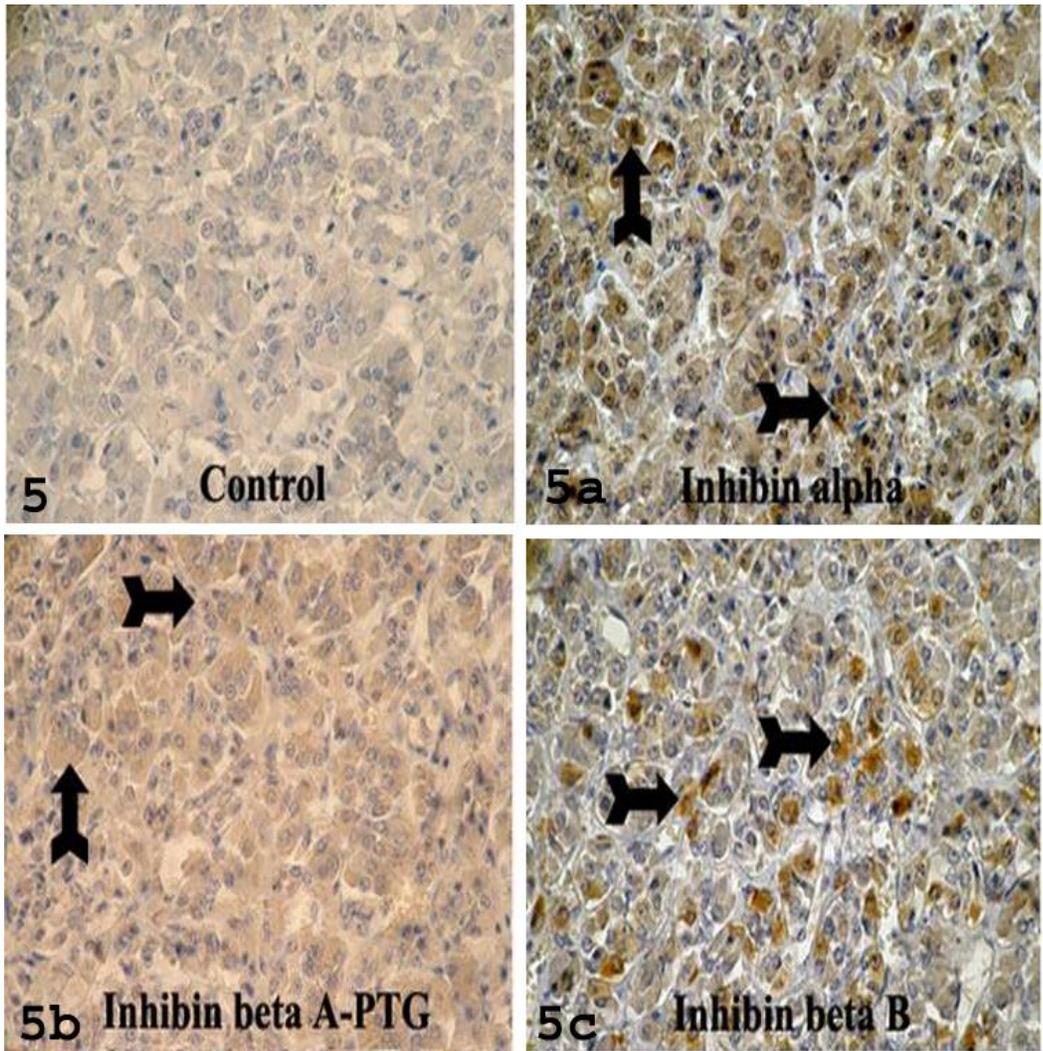
Plate (3): Photomicrograph of the buffalo-cows adenohypophysis at 1.5 years showing, control immunostaining (3), positive immunostaining specific for the inhibin α (3a), and negative immunostaining for βa (3b) and βb (3c) subunits (arrows).. X100

Plate (4): Photomicrograph of the buffalo-cows adenohypophysis at 4.5 years showing, control immunostaining (4), positive immunostaining specific for the inhibin α (4a), and weak positive immunostaining for βa (4b) and βb (4c) subunits (arrow heads). X400
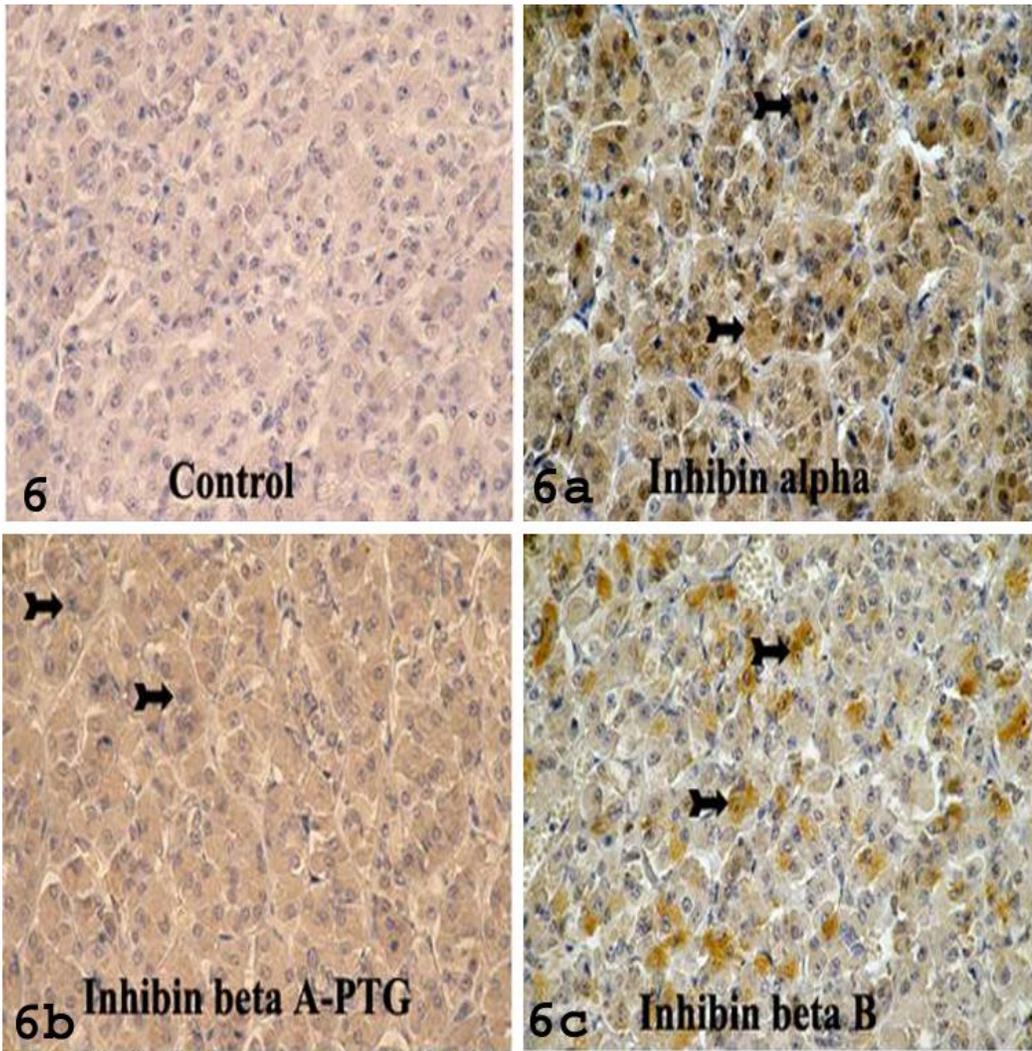
Plate (5): Photomicrograph of the buffalo-cows adenohypophysis at 8 years showing, control immunostaining (5), positive immunostaining specific for the inhibin α (5a), and βb (5c) and weak positive immunostaining for βa (5b) and subunits(arrows). X400

Plate (6): Photomicrograph of the buffalo-cows adenohypophysis at 12 years showing, control immunostaining (6), positive immunostaining specific for the inhibin α (6a), βb (6c) and weak positive immunostaining for βa (6b) subunits (arrows). X400
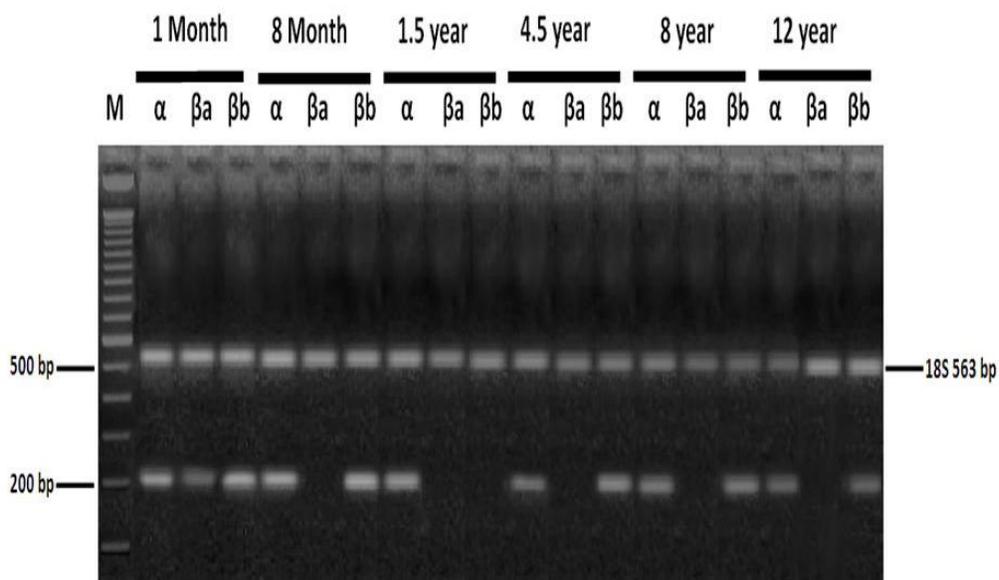
Figure (7): PCR products of 18S r RNA (563 b p), 217 bp of inhibin α, 209 bp of inhibin βa, and 218 bp of inhibin βb of six ages of Egyptian buffalo Bubalus bubalis L run on 2% agarose gel electrophoresis. (M) 100 bp DNA ladder.

Table (1): Primers sequence and product size of tested genes.

Primer name	Sequence	Expected PCR product
18S-rDNA(F)	5'-CCGGCGGCTTTGGTGACTC-3'	563 bp
18s-rDNA(R)	5'-GCTCGGGCCTGCTTTGAACA3'	
In-α (F)	5'- GGGACTGGACAGACAGGAG3'	217 bp
In- α (R)	5'-GGTGGAGCAGGAACAGAGAG-3'	
In-βa (F)	5'-GGAAGGGAAGAAGAGGGATG-3'	209 bp
In-βa (R)	5'-AGTCATTCCAGCCAATGTCC-3'	
In-βb (F)	5'-GCTGGAACGACTGGATCATT-3'	218 bp
In-βb (R)	5'-CTCGTCGTCGAAGTAGAGCA-3'	