THESIS

LOCALIZATION OF NERVE GROWTH FACTOR-β IN THE STALLION REPRODUCTIVE TRACT

Submitted by

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ABSTRACT

LOCALIZATION OF NERVE GROWTH FACTOR-β IN THE STALLION REPRODUCTIVE TRACT

Nerve growth factor-β (NGF-β) is a protein produced in the reproductive tract of camelids (camels, llamas, and alpacas) that has been identified as the ovulation inducing factor in seminal plasma. NGF-β from seminal plasma deposited into the reproductive tract of the female camelid acts systemically to stimulate secretion of luteinizing hormone (LH) from the anterior pituitary, which in turn induces follicle maturation and ovulation. The objectives of the present study were to 1) determine if NGF-β is present in the reproductive tract of the stallion and 2) identify the specific site(s) of production. The hypotheses were that NGF-β would be present in the stallion reproductive tract and would primarily be localized in Sertoli cells of the testis and the prostate gland. Immunohistochemistry on paraffin embedded paraformaldehyde fixed tissues was performed using a rabbit polyclonal anti-NGF-β antibody on a total of six male equine reproductive tracts, including a one-day old colt, a one-year old colt and 4 adult stallion tracts. Strong immunostaining was observed in the efferent ducts of the testis and the epithelial cells of the prostate, seminal vesicles, bulbourethral glands and ampullae. Weaker NGF-β staining was noted in Leydig cells, Sertoli cells and spermatogonia within the testes and in epithelial cells of the epididymis. In conclusion, immunohistochemistry revealed that NGF-β is present in the stallion reproductive tract and the protein is primarily present in the efferent ducts of the testes and in all accessory sex glands.

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CHAPTER 1

REVIEW OF LITERATURE

Physiology of Ovulation

Ovulation is a complicated process that culminates in release of an oocyte from the dominant follicle and subsequent transition of follicular tissue into luteal tissue (Senger, 2003). Ovulation is controlled by a complex neuroendocrine mechanism which includes signaling pathways between the ovary, hypothalamus and pituitary. Increased estradiol production by the developing dominant follicle during estrus stimulates an increase in the pulsatile secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus. GnRH is released into the hypothalamic-hypophysial portal system and travels to the anterior pituitary gland where it binds to GnRH receptors on gonadotroph cells and subsequently stimulates pulsatile releases of LH into the systemic circulation (Karsch, 1987). A progressive increase in the frequency of GnRH-induced LH pulses results in what is termed a 'surge' of LH, which ultimately leads to follicle maturation and ovulation.

The actual physical process of ovulation includes purposeful destruction of follicular tissue set in motion by the LH surge (Senger, 2003). The surge causes a series of biochemical events including gap junction breakdown between the granulosa cells and oocyte, an increase in production of prostaglandin E_2 (PGE_2) and prostaglandin F2α ($PGF_{2\alpha}$), blood flow increase to the ovary and dominant follicle, and there is a shift from estradiol (E_2) production to

progesterone (P_4) by the dominant follicle (Senger, 2003). The elevated blood flow ensures that the preovulatory follicle receives the essential hormonal and metabolic ingredients for final maturation. The progesterone is produced locally from the theca interna cells and it stimulates the synthesis of collagenase. This enzyme breaks down the collagen in connective tissue of the tunica albuginea. Prostaglandin $F_{2\alpha}$ causes contractions of ovarian smooth muscle. It also causes lysosomes to rupture and release their enzymes that further break down the connective tissue. The combination of ovarian smooth muscle contraction and the weakening of the follicle wall leads to ovulation.

Ovulation in mammals can be categorized into either spontaneous ovulation or induced (reflex) ovulation (Senger, 2003). Spontaneous ovulating species such as horses, cattle and sheep exhibit regular repeated estrous cycles that culminate in ovulation at or near the end of behavioral estrus, with or without being mated by a male. Ovulation in spontaneous ovulators is stimulated by an endogenous GnRH-induced LH surge.

Induced ovulating species such as camelids, rabbits, felines, ferrets, and mink have historically been thought to require copulation or physical stimulation of the vagina and/or cervix in order for ovulation to occur (San Martin et al., 1968; Senger, 2003; Silva et al., 2011). The prevailing hypothesis has been that stimulation of the sensory nerves in the vagina and/or cervix above a certain threshold will induce a surge of LH release from the anterior pituitary. The threshold for stimulation is achieved by either a prolonged copulation period (i.e. camels) or frequent repeated copulations (i.e. felids). Induced ovulators have regular, repeated waves of follicular development and regression, with each follicular wave associated with a rise in estrogen (estradiol-17β) levels (Senger, 2003). The elevated concentration of estrogens prime

the hypothalamus and pituitary for release of GnRH and LH, respectively, upon vaginal/cervical stimulation.

Camelids were historically believed to be classic induced ovulators dependent on vaginal/cervical stimulation for induction of an LH surge that would result in ovulation. However, recent studies have indicated that camelids are a 'modified induced ovulator' (Senger, 2003). The infusion of seminal plasma into the female reproductive tract of camelids during mating plays a crucial role in the induction of ovulation, more than tactile stimulation alone (Senger, 2003). Copulation alone in the absence of seminal plasma does not cause ovulation in llamas (Berland et al., 2016). A study used urethrostomized male llamas to test this theory as the males were able to mate naturally without intrauterine ejaculation. The urethrostomized males were unable to cause any ovulations despite the physical stimulus from copulation (Berland et al., 2016). It was concluded that llamas can be induced to ovulate by seminal plasma in the absence of copulation and that copulation alone cannot elicit ovulation in the absence of seminal plasma. Ultimately, it was determined that a substance in the semen of camelids, termed ovulation-inducing factor (OIF) is the substance that is responsible for the stimulation of ovulation.

Ovulation Inducing Factor (OIF)

History of OIF

The fluid portion of semen, known as seminal plasma, is largely secreted from the male accessory sex glands, with a lesser portion contributed by the epididymides and testes. The accessory gland system varies considerably among species and can differ by size, components

and volume of secretions, as well as anatomical and histological structure (Mann, 1964). The volume and constituent contribution of each accessory sex gland to the ejaculate is not well defined. Seminal plasma has been reported to affect spermatozoa transport, motility, and capacitation, as well as stimulate uterine contractions (Mann, 1964). A more recent discovery has been the role of ovulation-inducing factor (OIF) in seminal plasma to induce ovulation in camelids.

As noted previously, ovulation in induced ovulators was historically believed to be triggered by mechanical stimulation during copulation. The phenomenon of induced ovulation was first reported in South American camelids (San Martin et al., 1968; England et al., 1969) and later in Bactrian camels (Chen and Yuan, 1980). In llamas and alpacas, ovulation occurred in >95 % of females after mounting and penile intromission, while <14 % of females ovulated when intromission was not allowed (San Martin et al., 1968; England et al., 1969). Authors of a later study concluded that penile intromission was necessary to provide an adequate stimulation for LH release and subsequent ovulation in the alpaca (Fernandez-Baca et al., 1970). Collectively, these studies corroborated the concept that physical stimulation was the primary trigger for inducing ovulation in new world camelids.

In 1980, researchers in China reported that over 75 % of female Bactrian camels ovulated after intravaginal infusion of Bactrian seminal plasma (Chen et al., 1985). Results of this study indicated that there was a factor in seminal plasma that was responsible for induction of ovulation and not physical stimulation from copulation. Subsequent experiments in alpacas and llamas found that intramuscular administration of seminal plasma caused a surge of LH and induced ovulation in greater than 90 % of females treated (Adams et al., 2005). The

authors coined the term ‘ovulation inducing factor’ (OIF) for the as yet unknown substance in seminal plasma responsible for the effect.

Chemical Identity of OIF

Ovulation inducing factor was originally believed to be a peptide related to GnRH due to its LH-releasing effect on pituitary cells. However, anti-GnRH antibodies added to *in vitro* rat pituitary cell cultures did not inhibit the LH release from alpaca seminal plasma (Paolicchi et al., 1999). Studies on Bactrian camels suggested that OIF was a peptide consisting of different molecules ranging from 16 to 54 kDa and had GnRH-like bioactivity (Pan et al., 2001). OIF purified from camel seminal plasma caused both LH and FSH release from pituitary tissues and induced superovulation in mice. In addition, it was determined that OIF was structurally different from the native LHRH, LH, hCG, PMSG and PGF2α (Pan et al., 2001).

A series of experiments were conducted to define biochemical characteristics of OIF using molecular mass cut-off filtration, treatments with proteinase k, charcoal-dextran, or heat, and treatment with pronase E (Ratto et al., 2010). The fractions that resulted from the treatments were used in a llama bioassay. Results indicated that OIF was a protein molecule that is resistant to heat and enzymatic digestion with proteinase K, and has a molecular mass of approximately equal or higher than 30 kDa (Ratto et al., 2010). Protein fractions from llama seminal plasma were subsequently isolated and purified by liquid chromatography and administered to female llamas in an ovulation bioassay. Intramuscular administration of one purified protein fraction (C2) stimulated a surge of LH and induced ovulation in female llamas similar to the effects observed after intramuscular administration whole seminal plasma (Ratto

et al., 2011). The authors determined that OIF was a 14 kDa protein molecule that has an endocrine effect at the hypothalamus or pituitary. The identity of ovulation-inducing factor was discovered in a study testing the hypothesis that OIF is a single distinct and widely conserved entity (Ratto et al., 2012). Bull and llama seminal plasma was used to represent induced and spontaneous ovulators, respectively. The protein fraction from the previous study (Ratto et al., 2011) was isolated and MALDI-TOF revealed a molecular mass of 13,221 Da. Amino acid sequences of OIF were found to be homologous with human, porcine, bovine, and murine sequences of nerve growth factor-β (NGF-β). The full structure and sequence of OIF was determined to be identical to NGF-β by X-ray diffraction (Ratto et al., 2012). Nerve growth factor-like properties of OIF were observed and confirmed with neurite development and up-regulation of trkA in pheochromocytoma (PC_{12}) cells, which is a specific bioassay for NGF. Western blot analysis of bull and llama seminal plasma confirmed immuno-recognition of OIF using polyclonal mouse anti-NGF and NGF-β from mouse submandibular glands induced ovulation in llamas. The authors concluded that purified OIF from llama seminal plasma was highly conserved and identical to NGF-β (Ratto et al., 2012).

Other researchers confirmed that NGF-β was major protein in alpaca seminal plasma using liquid chromatography mass spectrometry (Kershaw-Young et al., 2012). The authors reported that intramuscular administration of alpaca seminal plasma, human NGF-β and the GnRH agonist buserelin all induced ovulation in 80 % of female alpacas treated (Kershaw-Young et al., 2012).

Nerve Growth Factor-β

Physiology and Chemical Structure

Nerve growth factor belongs to a family of neurotropins that includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. All are basic secretory proteins that exist as homodimers and have a molecular mass of approximately 27 kDa (Kolbeck et al., 1994). Nerve growth factor is composed of 3 subunits: α, γ, and β. The α-subunit has no known physiological effect and the γ-subunit may play a role in the processing of pro-NGF. The β-subunit is entirely responsible for the NGF biological activity (Thoenen and Barde, 1980). NGF binds to 2 different receptors: p75 NGFR and trkA. P75 NGFR, also known as low affinity NGF receptor, is a membrane spanning receptor with rapid dissociation kinetics. It also has the ability to bind to the all of the other neurotropins in the same family as NGF (Dissen et al., 1996).

The original function reported for NGF was promotion of survival and growth of sensory (dorsal root) and sympathetic neurons, and cells the adrenal medulla (Angeletti and Bradshawt, 1971). NGF was initially detected in mouse sarcoma, cobra venom, guinea pig prostate, goldfish brain, and adult mouse mandibular salivary glands (Angeletti and Bradshawt, 1971; Thoenen and Barde, 1980). Nerve growth factor was also found in non-neural cells including female and male reproductive tissue (Harper and Thoenen, 1980; Shikata et al., 1984). Another rich source of NGF was bovine seminal plasma and it was thought to be produced by the vesicular glands (Harper and Thoenen, 1980). NGF was also detected in the prostate gland of guinea pigs, rabbits, and bulls (Harper and Thoenen, 1980; Shikata et al., 1984).

Reproductive Function of Nerve Growth Factor-β

A majority of the research and information on endocrine and ovarian effects of NGF was established in new world camelids (llamas and alpacas). Administration of seminal plasma to female llamas and alpacas by intramuscular and intrauterine routes was used in a series of experiments to determine if the biological effects of NGF/OIF was based on local or systemic effects (Adams et al., 2005; Ratto et al., 2005). Adams and colleagues (2005) evaluated the seminal plasma of alpacas and llamas for the presence of an ovulation-inducing factor as well as the effect of OIF on pituitary function (Ratto et al., 2005). Intramuscular administration resulted in ovulation in 28 of 30 (93%) llamas and alpacas. No ovulations were detected in the intrauterine treatment group. A surge of LH was detected beginning 15 minutes and 75 minutes after intramuscular administration of GnRH and seminal plasma, respectively. It was concluded that the mechanism of action of OIF involved stimulation of a preovulatory surge in LH. The duration of the LH surge from the seminal plasma was significantly greater and more prolonged compared to the LH surge of GnRH treated animals. Plasma LH levels did not decline to pretreatment levels even after 8 hours after seminal plasma administration (Adams et al., 2005). The LH surge induced by intramuscular seminal plasma treatment was similar to the LH surge induced after a natural mating indicating an endocrine route of action for OIF. A follow-up study focused on the differences in biological responses between intramuscular and intrauterine administration of seminal plasma in camelids (Ratto et al., 2005). The hypothesis was that differences in effect were due to the attenuated absorption of OIF from the genital mucosa when infused into the uterus versus greater absorption following natural copulation. Copulation in camelids is a prolonged event lasting 30 to 50 minutes (San Martin et al., 1968)

and ejaculation is directly into the uterus. The prolonged copulation results in acute, transient inflammation of the endometrium from the repeated abrasion by the penis (Bravo et al., 1996). To mimic a more natural mating, researchers increased the dose of seminal plasma and infused the dose after endometrial curettage to simulate the abrasive effects of copulation (Ratto et al., 2005). Seven of 17 (41 %) females ovulated following intrauterine infusion of seminal plasma alone, whereas 10 of 15 (67%) females ovulated after infusion of seminal plasma along with endometrial curettage. None of the female alpacas ovulated after intrauterine administration of a saline placebo with or without endometrial curettage. In that study, intramuscular administration was still more effective and caused more ovulations (93 %) than the intrauterine route. This study concluded that OIF from seminal plasma exerts its effects systemically rather than locally and disruption of the endometrial mucosa by curettage aided in the absorption and biological activity of OIF. Most importantly, it was determined that ovulation in alpacas is not due solely to physical stimulation of the genital tract, but that OIF in seminal plasma is needed for induction of ovulation (Ratto et al., 2005).

Other Induced Ovulators

Although majority of the research was done in new world camelids, NGF is not restricted to only those species. The first discoveries of OIF were in Bactrian camels. Nerve growth factor-β has also been found in high abundance in Dromedary camel seminal plasma (Meriem et al., 2017). Intramuscular injection of NGFβ isolated from Dromedary seminal plasma induced ovulation at a similar rate compared to buserelin treatment (Meriem et al., 2017).

Other induced ovulatory species may also have NGFβ. Johnston et al. (2004) reported that koalas exhibit a semen-induced luteal phase (Johnston et al., 2004). A total of 77 % of female koalas had an induced luteal phase with both semen deposition in the urogenital sinus and physical stimulation of the urogenital sinus. Semen deposition alone caused a luteal phase in 44 % of females whereas none of the koalas ovulated after physical stimulation alone. It was concluded that semen or OIF must be involved in the induction of a luteal phase or ovulation in the koala (Johnston et al., 2004).

Rabbit seminal plasma also has ovulation inducing factor. Intramuscular administration of rabbit seminal plasma induced ovulation in 100 % of female llamas, but there was little effect on female rabbits when they were administered either llama or rabbit seminal plasma intramuscularly (Silva et al., 2011). OIF may not play as crucial a role in inducing ovulation in rabbits as it does in camelids.

Spontaneous Ovulators

The presence of an ovulation-inducing factor may not be exclusive to species that are induced ovulators. The first spontaneous ovulator species reported to have NGF-β in their semen was cattle were it was purified from the seminal plasma of bulls (Harper et al., 1982). A comparative study was done to determine if the seminal plasma of bulls would induce ovulation in llamas (Ratto et al., 2006). Intramuscular administration of bovine seminal plasma caused 26 % of female llamas to ovulate. Purified OIF/NGF from llama seminal plasma administered to prepubertal heifers accelerated regression of the dominant follicle and initiated a new follicular wave (Tanco et al., 2012; Adams et al., 2016). It was suggested that OIF

may play a role follicular wave dynamics by suppressing the dominant follicle. Administration of bovine seminal plasma along with LH to heifers resulted in more synchronous ovulations along with a luteotrophic effect (Tribulo et al., 2015).

There are few published reports of OIF/NGF in other spontaneous ovulatory species. One study evaluated the effect of equine and porcine seminal plasma on induction of ovulation in llamas (Bogle et al., 2009; Bogle et al., 2011). Equine seminal plasma stimulated ovulation in 38 % of llamas with a low dose (3 ml) and 22 % of llamas with a higher dose (8 ml) with intramuscular administration. Porcine seminal plasma was not as successful as equine seminal plasma, with no ovulations induced in female llamas following administration of a low dose (3 ml) and a 33 % ovulation rate in llamas after administration of a high dose (10 ml). However, the actual amount of NGF in the stallion or boar seminal plasma was not determined. The authors concluded that OIF is present in both equine and porcine seminal plasma (Bogle et al., 2011).

Another study evaluated the presence of NGF-β in the seminal plasma of boars, bulls, rams, stallions, alpacas, and camels (Druart et al., 2013). Samples were analyzed using 2DLC MS/MS to determine if they contained NGF-β (Druart et al., 2013). Seminal plasma from camelids and cattle had NGF-β in high abundance as detected in both western blot and 2DLC MS/MS. In contrast, NGF-β was only detected in rams and stallions by mass spectroscopy (Druart et al., 2013). There was no evidence of OIF/NGF in porcine seminal plasma.

Source of NGF in the Male Reproductive Tract

NGF-β was first discovered in the prostate gland of the guinea pig (Harper and Thoenen, 1980; Shikata et al., 1984)**.** Accessory sex glands of mice, rats, guinea pigs, hamsters, rabbits, humans, and bulls were all evaluated for NGF with a biological assay and a two-site radioimmunoassay, but only the prostate glands of the guinea pig, rabbit, and bull were noted to contain NGF (Harper and Thoenen, 1980). This research lead to studies in other species including llamas, rats, cattle, bison, elk, and white-tailed deer (Bogle et al., 2018). NGF-β was localized in at least one accessory sex gland in all of the species analyzed. The principal source of NGF-β in the reproductive tissues varies by species. In the llama, the glandular epithelium of the body and disseminate prostate are the main sources (Bogle et al., 2018). In cattle and bison, immunostaining for NGF-β was noted to be strongest in the vesicular glands and ampullae (Bogle et al., 2018). In elk and white-tailed deer, immunostaining for NGF-β was primarily localized to the ampullae and prostate (Bogle et al., 2018).

Stallion Anatomy and Physiology

The stallion reproductive anatomy consists of two testes, two epididymides, two vas deferens, two ampullae, paired vesicular glands, a prostate gland, and a bulbourethral gland (Amann, 2011). The testis is responsible for spermatogenesis and the production of testosterone. The lumen of seminiferous tubules within the testes are lined with Sertoli cells and germ cells and is the location of spermatogenesis. The blood-testis barrier, composed of tight junctions between the plasma membranes of adjacent Sertoli cells, isolates the advanced germ cells from the immune system of the host. The interstitial tissue contains blood vessels,

lymphatic channels, nerves, connective tissue, and Leydig cells. The Leydig cells are responsible for testosterone production. The seminiferous tubules empty into the rete testis which is connected to the efferent ducts.

The epididymis consists of one long, coiled tubule and is composed of 3 different parts: the head, body, and tail. The efferent ducts from the testes lead into the head of the epididymis. A majority of fluid entering the epididymis from the testis is absorbed in the head of the epididymis. The epididymis has a secretory effect as well. The epididymal epithelium has been reported to secrete 117 proteins with two of the major ones being lactoferrin and clusterin (Amann, 2011). The body of the epididymis is involved with sperm maturation that is dictated by specific secretions of the epithelium. The main function of the tail of the epididymis is storage of spermatozoa. The tail of the epididymis is continuous with the vas deferens or ductus deferens. A main function of the vas deferens is transport of spermatozoa into the pelvic urethra during ejaculation. The distal end of the vas deferens is lined with glandular tissue of the ampullae.

The accessory sex glands of the stallion include the ampullae, vesicular glands, prostate gland, and bulbourethral gland. These glands produce a majority of the fluid portion of the ejaculate called the seminal plasma. The bulbourethral gland produces a majority of the pre-seminal fluid and contributes to the seminal plasma. The prostate gland produces fluid that helps cleanse the urethra prior to and during ejaculation. The seminal vesicles expand greatly during sexual stimulation and produce the gel fraction of the semen.

Potential Role of NGF in Stallions

There is limited research published on the presence or function of NGF-β in horses. NGF-β has been detected in in equine seminal plasma (Bogle et al., 2011; Druart et al., 2013). The location of production of NGF-β in the stallion reproductive tract is not known and is the subject of the current research.

Summary

In summary, NGF-β is a protein that is conserved across species that are classified as either spontaneous or induced ovulators. NGF-β could play a role in induction of ovulation in the mare like it does in camelids. If so, equine NGF-β could be synthesized and used as an alternative treatment for induction of ovulation in mares.

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CHAPTER 2

LOCALIZATION OF NERVE GROWTH FACTOR-β IN THE STALLION REPRODUCTIVE TRACT

Introduction

The fluid portion of semen, known as seminal plasma, is largely secreted from the accessory glands with a lesser portion contributed by the epididymides and testes. The stallion accessory gland system includes paired ampulla surrounding the vas deferens, paired seminal vesicles, a prostate gland, and a bulbourethral gland (Mann, 1964; Amann, 2011). The accessory gland system varies considerably among species. Bulls and stallions have all four accessory sex glands, whereas camelids do not have seminal vesicles (Mann, 1964; Adams et al., 2016). The glands can differ between species by size and secretions, as well as anatomical and histological structure (Mann, 1964). This variability can be seen in the characteristics of the ejaculate, but the contributions of each gland and its function are largely unknown. Seminal plasma has been reported to directly affect spermatozoa transport, motility and capacitation, as well as stimulation of uterine contractions in the female (Mann, 1964; Druart et al., 2013; Adams et al., 2016). Another physiologic function of seminal plasma recently reported is the induction of ovulation in female camelids by an ovulation-inducing factor (OIF) present in semen of male camelids (Chen et al., 1985; Xu et al., 1985; Pan et al., 1992).

Ovulation inducing factor was first observed in Bactrian camels (Chen et al., 1985). Ovulation occurred in female camels after the administration of seminal plasma by intravaginal, intramuscular, or intrauterine routes (Chen et al., 1985; Xu et al., 1985; Pan et al., 1992). The

same phenomenon was confirmed in llamas and alpacas where >90 % of females ovulated after an intramuscular dose of seminal plasma (Adams et al., 2005). The ovulation rate of female llamas following systemic administration of seminal plasma was similar to that of llamas treated with gonadotropin-releasing hormone (GnRH) (Cystorelin®, Merial Canada Inc., Victoriaville, PC, Canada) or following natural service. Seminal plasma treatment stimulated a surge of luteinizing hormone (LH) which was subsequently responsible for induction of ovulation (Adams et al., 2005). A luteotrophic effect was also observed in treated animals as plasma progesterone concentrations were higher, the corpus luteum (CL) tended to grow for a longer period, and the diameter of the CL was greater in the seminal plasma treatment group (Adams et al., 2005).

Ovulation-inducing factor has been determined to be structurally identical to the protein Nerve Growth Factor-β (NGF-β/NGF) (Ratto et al., 2012). NGF-β belongs to a family of neurotropins which typically exists in nature as a homodimer with a molecular mass of 26-27 kDa (Ratto et al., 2012; Adams et al., 2016). Nerve growth factor has an essential role in promoting survival, maintenance and growth of sensory (dorsal root) and sympathetic neurons and cells of the adrenal medulla (Harper and Thoenen, 1980; Adams et al., 2016).

NGF was originally identified in mouse sarcoma, cobra venom, and adult male mouse submaxillary glands (Harper and Thoenen, 1980; Thoenen and Barde, 1980; Adams et al., 2016), but was subsequently identified in reproductive tissue including the prostate gland of guinea pigs (Shikata et al., 1984). This discovery prompted research to determine if NGF-β was present in accessory sex glands of other species (Adams et al., 2016). Accessory sex glands of mice, rats, guinea pigs, hamsters, rabbits, humans, and bulls were evaluated for NGF, with a biological assay and a two-site radioimmunoassay, but only the prostate glands of the guinea pig, rabbit,

and bull were identified to contain NGF (Harper and Thoenen, 1980). After the discovery that ovulation inducing factor was structurally identical to nerve growth factor β, more species were studied including llamas, rats, cattle, bison, elk, and white-tailed deer (Bogle et al., 2018). In all species evaluated, NGF was localized in at least one male accessory sex gland, with the primary source being the prostate in llamas, vesicular gland and ampullae in bovids (cattle and bison), and the ampullae and prostate in cervids (elk and white-tailed deer) (Bogle et al., 2018).

Limited research has been performed on the presence or function of NGF-β in horses. Bogle and coworkers (2009) injected female llamas with equine seminal plasma, which caused between 22% to 38% of the llamas to ovulate, depending on dose (Bogle et al., 2009). Druart et al. (2013) identified NGF-β in seminal plasma from stallions using two-dimensional liquid chromatography-tandem mass spectrometry (2DLC MS/MS).

The objectives of the present study were to determine if NGF-β protein is present in the reproductive tract of the stallion and to identify the specific site(s) of production. The hypotheses were that NGF-β will be present in the stallion reproductive tract and production would primarily be localized in the Sertoli cells of the testis and in the prostate gland.

Materials and Methods

Tissue Preparation

Reproductive tracts were harvested from 6 male horses that were euthanized for unrelated medical conditions at the Veterinary Teaching Hospital, Colorado State University. Tissues came from four adult stallions, one colt approximately one year of age and one colt foal that was one day old. Reproductive tissues were collected during necropsy and included testes,

head, body, and tail of the epididymis, vas deferens, ampullae, seminal vesicles, prostate, and bulbourethral gland. Alpaca testes and equine cornea were collected as positive control tissues. All tissues were fixed for at least 24 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS) and subsequently embedded in paraffin blocks. Tissues were then processed to obtain 5-µm paraffin sections on glass slides. Additional non-reproductive tract tissues harvested and processed included heart, lung, skeletal muscle, kidney, and spleen from an adult stallion.

Immunostaining

Slides containing tissue sections were washed three times in xylene and twice in absolute ethanol. Slides were then submerged in a successive gradient of ethanol solutions (90 %, 70 %, 50 %, and 30 %), followed by two washes with deionized water and then submerged in citrate buffer solution (pH 6.0) and incubated in a pressure cooker for 3 minutes. Slides were subsequently cooled to room temperature for 30 minutes and washed twice in deionized water. A general block (5 % normal goat serum in universal blocking solution made from bovine serum albumen) was applied to the tissue for 60 minutes followed by treatment with hydrogen peroxide blocking solution (0.05 % hydrogen peroxide) for 15 minutes. The primary antibody used for immunohistochemical localization of NGF was polyclonal rabbit antiserum raised against human NGF (PA5-14872; Thermo Fisher Scientific, Waltham, MA). The antibody was diluted 1:100 in antibody diluent (64211; Abcam, Cambridge, United Kingdom). Slides were incubated overnight at 4° C then washed three times for 5 minutes with wash buffer solution (1% TBS and 0.005 % Triton™ X in deionized water). Tissues were then incubated in a humidified chamber with the secondary antibody (goat anti-rabbit IgG-HRP; G-21234; Thermo Fisher

Scientific, Waltham, MA) diluted 1:500 in antibody diluent (64211; Abcam, Cambridge, United Kingdom). A 3,3' diaminobenzidine (DAB) substrate kit (64238; Abcam, Cambridge, United Kingdom) was used for color development and hematoxylin used as a counter stain. Slides incubated with antibody diluent but without the primary antibody served as negative controls. A NGF-β protein block was applied to all reproductive tissue slides with the primary antibody to test the specificity of the antibody. Slides containing equine cornea and alpaca testes were used as positive controls. All test tissues and controls were stained simultaneously for controlled timeframes to ensure uniformity of staining. Histology was performed on serial sections stained with hematoxylin and eosin to evaluate morphology.

Results

NGF-β was identified in both positive control tissues (equine cornea and alpaca testis; Figure 1) and tissues from all 6 male equine reproductive tracts. Negative controls reacted appropriately. Tissues from all reproductive organs exhibited positive immunostaining, but staining intensity for NGF-β differed between tissues (Table 1). In the testes, the strongest staining was in the efferent ducts and lighter staining was observed in interstitial (Leydig) cells, Sertoli cells and spermatogonia (Figure 2). In the interstitial cells, a majority of immunostaining was located in the apical region, with more limited immunostaining in the nucleus and cytoplasm. The head, body, and tail of the epididymis, as well as the vas deferens, exhibited apical immunostaining of the epithelial cells (Figure 3). Intensity of staining increased distally with the strongest immunostaining observed in the tail of the epididymis and vas deferens. Tails of spermatozoa in the body region of the epididymis also appeared to stain positive for NGF.

Accessory sex gland tissue exhibited more staining than tissues from the other reproductive organs aside from the efferent ducts of the testes. The ampullae, seminal vesicles, prostate and bulbourethral glands all exhibited strong apical staining (Figure 4). The disseminate and body of the prostate gland stained with the same intensity. The strongest apical staining was observed in the prostate and seminal vesicles. The prostate and bulbourethral glands also exhibited cytoplasmic staining along with limited staining of the nucleus and smooth muscle.

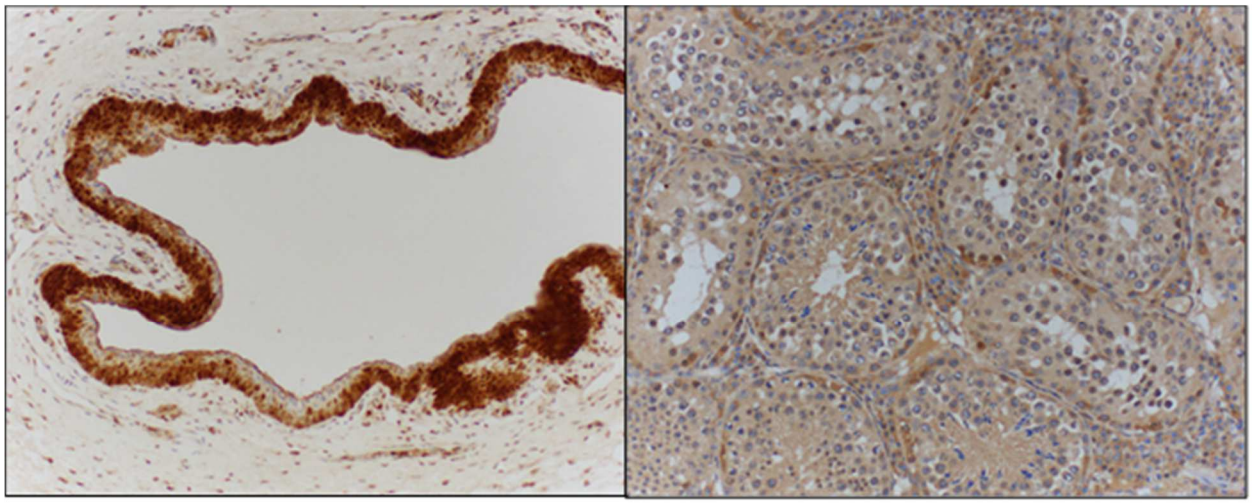

Figure 1. Staining of positive control tissues including equine cornea (left image) and alpaca testes (right image).

Table 1. NGF-β staining intensity in the four adult stallion reproductive tracts. Relative staining intensities were graded + (weak to moderate), ++ (strong), +++ (very strong).

Reproductive Tissue	NGF-β Staining Intensity
Sertoli Cells of Testes	+
Leydig Cells of Testes (interstitial)	+
Efferent Duct of Testes	+++
Head of Epididymis	+
Body of Epididymis	+
Tail of Epididymis	++
Vas Deferens	++
Ampulla	+++
Seminal Vesicle	+++
Prostate Gland	+++
Bulbourethral Gland	+++

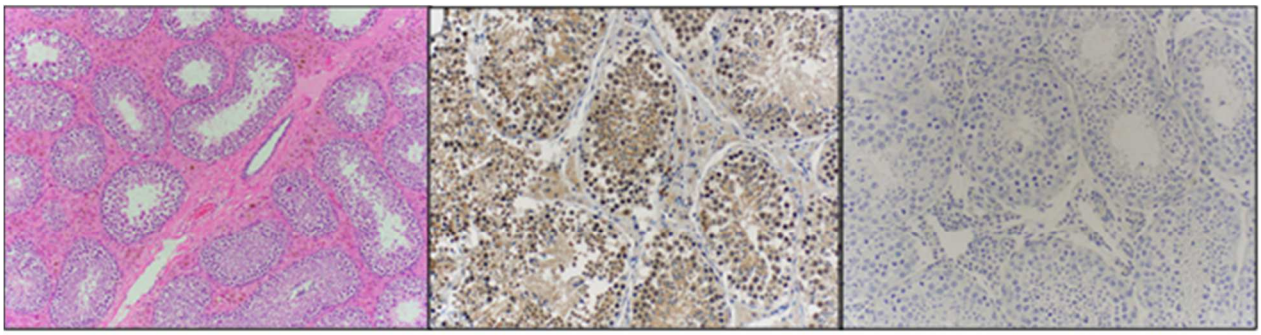

Figure 2. Testicular tissue stained with hematoxylin and eosin (left image), immunostained for NGF-β (middle image), and negative control (left image).

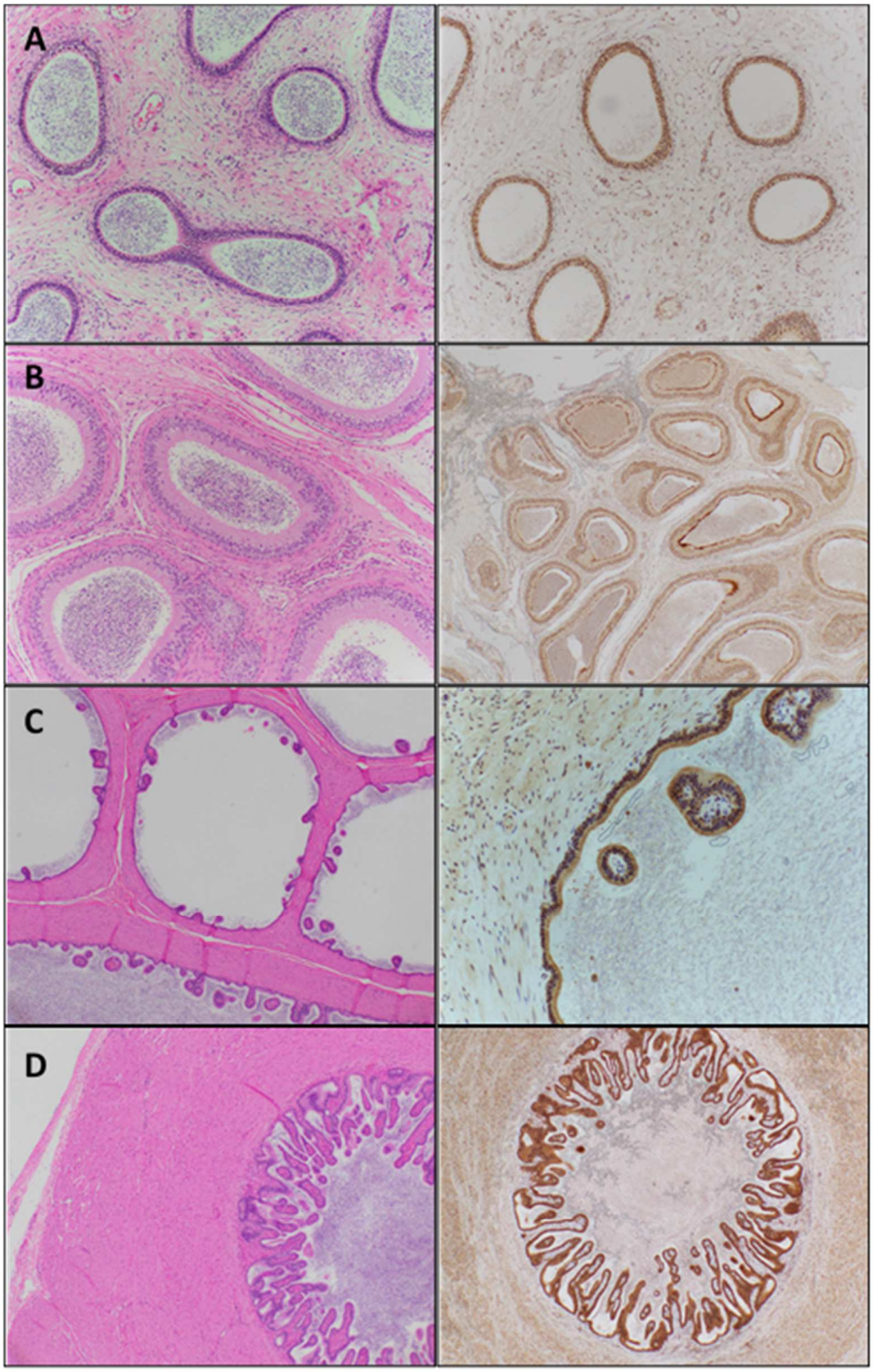

Figure 3. Epididymal tissue stained with hematoxylin and eosin (left image) and immunostained for NGF-β (right image). A=head of epididymis, B=body of epididymis, C=tail of epididymis, D=vas deferens.

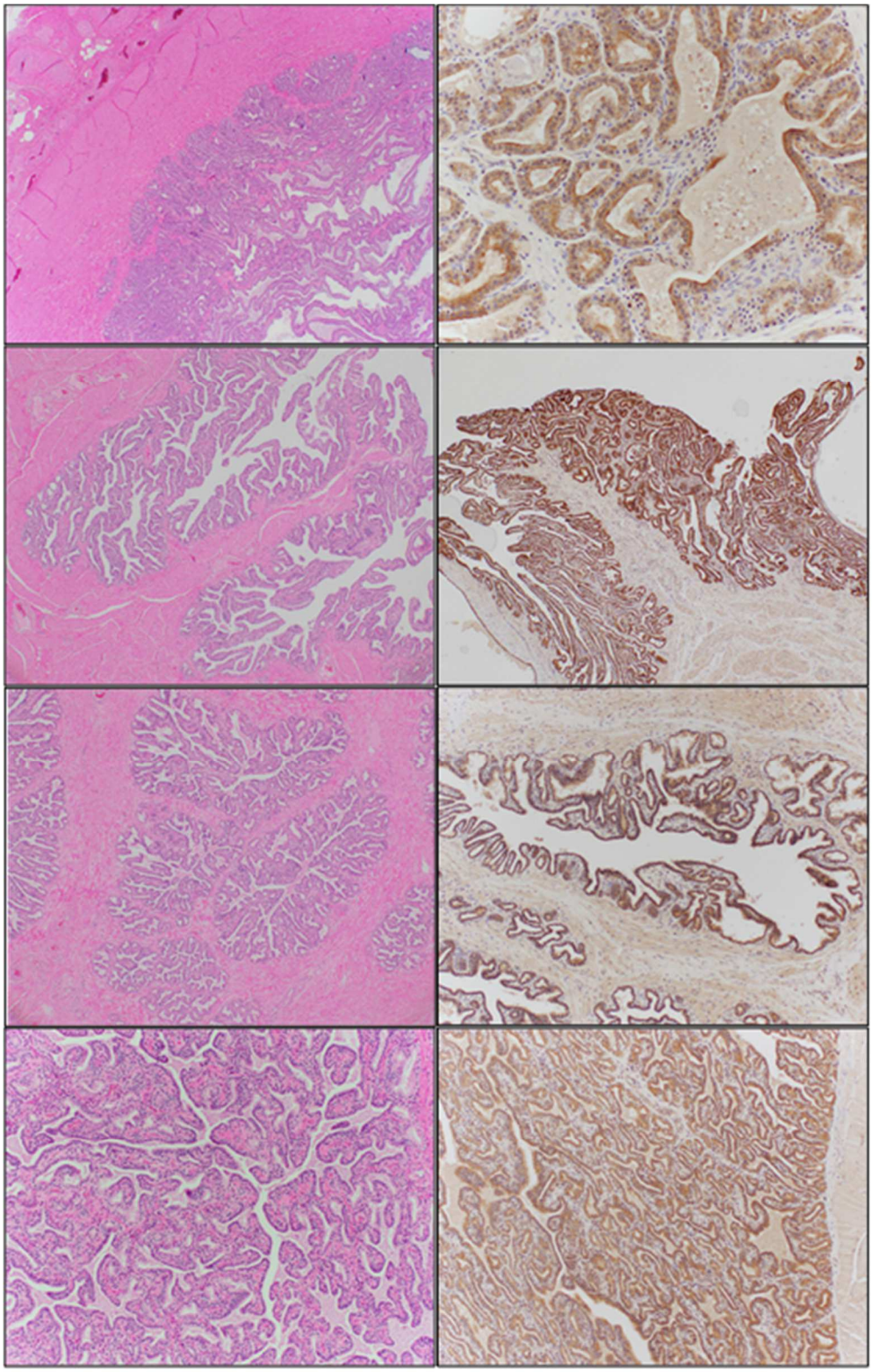

Figure 4. Accessory glands stained with hematoxylin and eosin (left image) and immunostained for NGF-β (right image). A=ampulla, B=seminal vesicle, C=prostate, and D=bulbourethral gland.

No differences in immunostaining of NGF were noticed between the one-day-old foal, the one-year-old colt, or the adult reproductive tracts. Immunostaining for NGF-β in the non-reproductive tract tissues including heart, lung, spleen, skeletal muscle, and kidney were all negative except for the lung. Positive staining was observed in the bronchial epithelial cells (Figure 5). The NGF-β protein block, evaluated on all of the reproductive tissues, resulted in complete blockage of immunostaining in all tissues with the exception of only a limited blockage in testicular tissue.

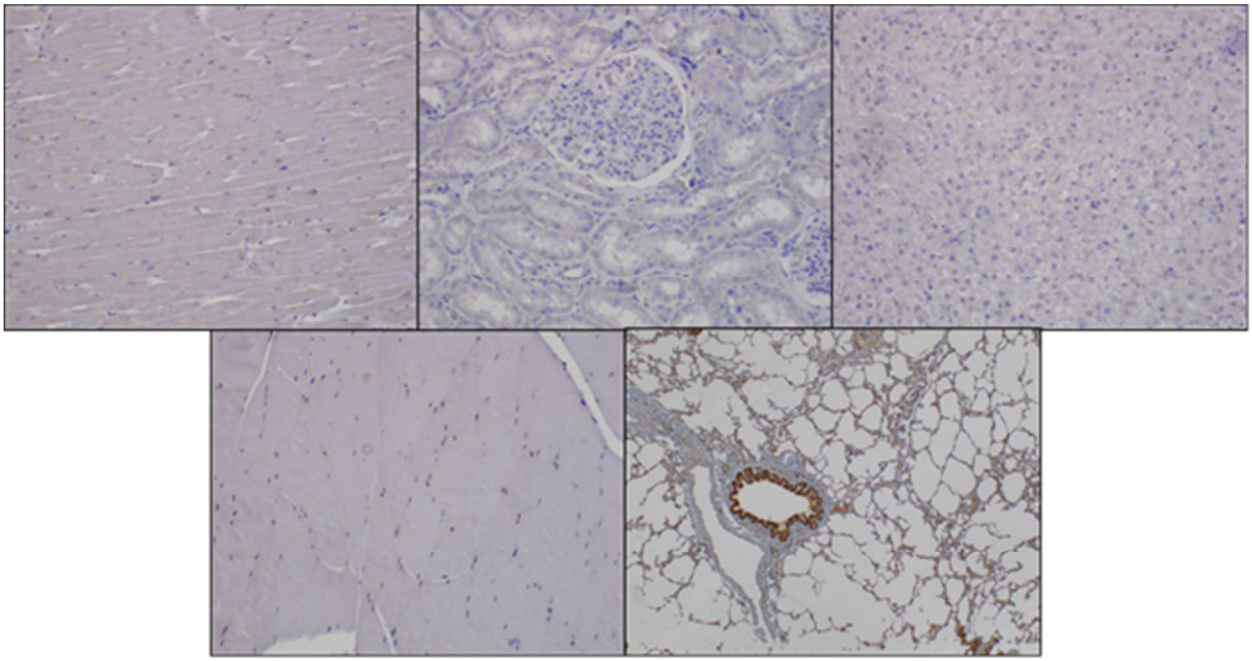

Figure 5. Non-reproductive tract tissues stained for NGF-β; heart (top left image), kidney (top middle image), liver (top right image), skeletal muscle (bottom left image), and lung (bottom right image).

Discussion

Our original hypotheses were that NGF-β would be found in the stallion reproductive tract and that it would primarily localize in the Sertoli cells of the testes and the prostate gland. NGF-β immunostaining was observed in multiple tissues within the reproductive tract of

stallions, but was primarily localized in the efferent ducts of the testes, as well as the ampullae, seminal vesicles, prostate gland, and the bulbourethral gland.

The principal source of NGF-β in reproductive tract tissues varies by species. In the llama, the glandular epithelium of body and disseminate parts of the prostate were the main sources (Bogle et al., 2018). In cattle and bison, immunostaining for NGF-β was noted to be strongest in the vesicular glands and ampullae (Bogle et al., 2018). In elk and white-tailed deer, immunostaining for NGF-β was primarily localized to the ampullae and prostate (Bogle et al., 2018).

Stallion testes exhibited the strongest immunostaining for NGF-β in the efferent ducts, with light, scattered immunostaining noted in the interstitial cells, Sertoli cells, spermatogonia, and smooth muscle. In the bull testis, immunoreactivity was localized in the myoid epithelial cells surrounding the seminiferous tubules, whereas spermatids and interstitial cells were negative for NGF-β staining (Bogle et al., 2018). In camelids and cervids, low intensity staining was noted in the interstitial cells, smooth muscle, and connective tissue within the testis (Bogle et al., 2018).

In the present study, no differences in immunostaining for NGF-β was noted between testicular tissue of male horses of different ages. In a study of age-associated NGF-β immunostaining in alpacas, Wang and colleagues (2011) noted positive immunostaining in the Sertoli cells, spermatogonia, and primary spermatocytes of 1-month old cria, whereas the 12-month old male alpaca showed strongest staining in the spermatogenic cells, Sertoli cells, and in scattered interstitial cells (Wang et al., 2011). Testes from a 24-month old alpaca stained

positive in the stromal cells, Sertoli cells, and germ cells, but the intensity was less than that of the 12-month old alpaca (Wang et al., 2011). It has been suggested that NGF-β could play a role in pubertal development of the testes and spermatogonia of the male alpaca (Wang et al., 2011).

Epididymal tissue of the stallion exhibited strong immunostaining in the apical region of the epithelium throughout the head, body and tail. The intensity of the immunostaining increased distally from the head of the epididymis to the tail of the epididymis, with the strongest staining located in the epididymal tail and continuing into the vas deferens. Cytoplasmic and nuclear staining of the epithelium and some scattered and nonspecific staining were observed in the smooth muscle of the epididymis. Bulls have also been reported to exhibit immunostaining for NGF-β in the epithelium of the epididymis (Bogle et al., 2018). Camelids, cervids and rats were noted to exhibit faint to strong immunostaining in the connective tissue of the epididymis (Bogle et al., 2018).

The prostate gland has been reported to exhibit immunostaining for NGF in llamas, guinea pigs, rabbits, and cattle (Harper and Thoenen, 1980; Shikata et al., 1984; Bogle et al., 2018). In the llama, the strongest immunoreactivity was in the glandular epithelium of the body and disseminate parts of the prostate. In the current study, the stallion exhibited strong epithelial staining in both the body and disseminate prostate as well as all of the other accessory sex glands. Bulls have been reported to exhibit immunostaining for NGF-β in the epithelium of all of their accessory glands, with the exception of the bulbourethral gland (Harper and Thoenen, 1980; Bogle et al., 2018). To date, the stallion is one of the only species noted to have NGF-β immunostaining in the epithelium of the bulbourethral gland.

Strong epithelial staining throughout the accessory sex glands of the stallion suggests that NGF-β is secreted into the seminal plasma. In camelids, NGF-β from the male reproductive tract is secreted into the seminal plasma and has a physiologic role in inducing ovulation in the female (Pan et al., 2001; Meriem et al., 2017). Administration of NGF-β to llamas and alpacas by intramuscular injection or infusion into the vagina or uterus stimulates a surge of luteinizing hormone (LH) which subsequently induced follicular maturation and ovulation (Adams et al., 2005; Ratto et al., 2005). In the cow, a spontaneous ovulator, NGF derived from bull seminal plasma was reported to improve synchronization of ovulation and enhanced luteal development (Tribulo et al., 2015). Mares are another spontaneous ovulator; however, mares exhibit a broad surge of LH during estrus (Adams and Bosu, 1988). It is not known if NGF-β within seminal plasma of stallions plays any physiologic role in the mare in modulation of gonadotropin secretion or induction of ovulation.

In conclusion, NGF-β was detected in multiple sites within the stallion reproductive tract. The primary locations of NGF-β immunoreactivity were the efferent ducts of the testes, and the apical epithelium of all four accessory sex glands.

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CHAPTER 3

SUMMARY

Ovulation inducing factor (OIF) is a component of seminal plasma that can induce ovulation in female camelids by intramuscular, intravaginal, or intrauterine administration (Chen et al., 1985; Xu et al., 1985; Pan et al., 1992; Adams et al., 2005). OIF has been found to be structurally identical to the neurotropin nerve growth factor-β (NGF-β) (Ratto et al., 2012). OIF/NGF-β is conserved across species that can be classified as either spontaneous (cattle, bison, elk, or white-tailed deer) or induced ovulators (llamas, alpacas, camels, rabbits, or koalas) (Harper and Thoenen, 1980; Ratto et al., 2006; Bogle et al., 2018). Although, limited research has been investigated on the function or presence of NGF-β in horses. The goal of the thesis was to determine if NGF-β is present in the reproductive tract of the stallion and to identify where it is produced.

Immunohistochemistry confirmed that NGF-β is localized in multiple tissues in the stallion reproductive tract. It primarily stained in the efferent ducts of the testes, as well as the ampullae, seminal vesicles, prostate gland, and the bulbourethral gland. The strong epithelial staining throughout the accessory sex glands suggest that NGF-β is secreted into the seminal plasma from those locations.

Future studies should investigate the concentration or amount of NGF-β in the seminal plasma and determine the role it plays in the physiology of mares. If NGF-β induced ovulation in

the mare like it does in camelids, it could be synthesized and used as an alternative treatment for induction of ovulation in mares.

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APPENDIX 1

PHARMACOLOGICALLY INDUCED EJACULATION IN STALLIONS-CLINICAL EXPERIENCE AND FAILED ATTEMPTS TO IMPROVE SUCCESS RATE WITH OXYTOCIN AND PROSTAGLANDINS

Introduction

Semen is traditionally collected from stallions by use of an artificial vagina with the stallion mounting a mare or breeding phantom (Love, 1992; Pickett et al., 2000; Hurtgen , 2009). Traditional semen collection may not be appropriate for stallions with severe musculoskeletal issues, neurologic defects, penile paralysis, or behavioral issues (McDonnell, 1992; McDonnell, 2005; McDonnell, 2011). Alternatively, other techniques such as ground collection using an artificial vagina (Schumacher and Riddell, 1986; Forney and McDonnell, 1999; Meroni et al., 2012), manual stimulation (Crump and Crump, 1989; McDonnell and Love, 1990), and pharmacologically induced-ex copula ejaculation (McDonnell and Odian, 1994; Johnston and DeLuca, 1998) have been developed for stallions.

Pharmacologically induced ejaculation ('chemical ejaculation') can be accomplished by administration of imipramine hydrochloride alone (McDonnell et al., 1987) or xylazine hydrochloride alone (McDonnell and Love, 1991) or a combination of imipramine followed by xylazine (McDonnell and Odian, 1994; McDonnell, 2001). Imipramine is a tricyclic antidepressant drug used in human medicine in the management of depression (Azima and Vispo, 1959), nocturnal enuresis (Banerjee et al., 1993), retrograde ejaculation (Ochsenkühn et al., 1999), and premature ejaculation (Balon, 1996). Administration of imipramine is

hypothesized to 'lower the ejaculation threshold' of stallions (McDonnell, 1992; McDonnell, 2011). Xylazine hydrochloride is an α_2 adrenergic receptor agonist used primarily for sedation in horses. Administration of xylazine hydrochloride or other α_2 agonists such as detomidine hydrochloride (Rowley et al., 1999; Hurtgen JP, 2009) is occasionally associated with a side effect of inducing passive emission of semen during sedation. Other protocols for pharmacologic induction of ejaculation have used different combinations of detomidine, butorphanol tartrate, imipramine, xylazine, clomipramine, and/or prostaglandin $F_{2\alpha}$ (McDonnell, 1992; Turner et al., 1995; Rowley et al., 1999; McDonnell, 2001; McDonnell, 2011; Josson and Whitacre, 2012).

Semen samples obtained by pharmacologically induced-ex copula ejaculation are reported to be low in volume and high in spermatozoa concentration (McDonnell and Odian, 1994; Turner et al., 1995; McDonnell, 2001; McDonnell, 2011). Semen collected pharmacologically can be used for fresh insemination, cooled-transport, or may be cryopreserved for later use (Turner et al., 1995; Feary et al., 2005) and has been used successfully to obtain pregnancies in mares (Card et al., 1997; Feary et al., 2005).

The success rate of inducing ejaculation with imipramine and/or xylazine ranges from 33 to 68 % (McDonnell and Odian, 1994; Johnston and DeLuca, 1998; McDonnell, 2001), but is quite variable between stallions and between studies. The objectives of the present study were to: 1) document success rate of pharmacologically induced ejaculation of stallions in a clinical setting, and 2) determine if administration of oxytocin at the same time as xylazine or administration of a prostaglandin analogue prior to xylazine would enhance the success rate of

a standard imipramine-xylazine protocol for pharmacologic induction of ejaculation in aged stallions.

Materials and Methods

Clinical Experience

A standard protocol consisting of a single dose of imipramine hydrochloride (3.0 mg/kg) orally followed approximately one hour later by intravenous administration of xylazine hydrochloride (0.33 to 0.5 mg/kg) was used in attempts to pharmacologically induce ejaculation in stallions in the clinical equine reproduction program at Colorado State University. A retrospective investigation of the records on success of chemical ejaculation in 50 attempts on 12 stallions over a 6 year period were reviewed. Semen parameters for ejaculates collected an additional 22 normal stallions using an artificial vagina during the same time periods as the 'chemical ejaculation' stallions were recorded for comparative purposes.

Research Stallions

Seven Quarter Horse stallions were used in the study. These stallions ranged in age from 17 to 27 years (mean age was 22.7 ± 3.5 years), in weight from 430 to 530 kg and had body condition scores between 4 and 6, based on score index of 1 (poor) to 9 (extremely fat) (Henneke et al., 1983). Three of the stallions were housed indoors in box stalls with daily turn out into a paddock, while four stallions were housed outside in a paddock and brought inside into a box stall on treatment days. Clean-out collections were performed on all stallions prior to the onset of the experiment by artificial vagina. All attempts at pharmacologically inducing ejaculations were performed in 16 ft x 16 ft box stalls with only one person in the stall and

minimal outside disturbance. All procedures were approved by Institutional Animal Care and Use Committee.

Traditional Chemical Ejaculation

Research stallions were administered a standard chemical ejaculation protocol consisting of a single oral dose of imipramine hydrochloride (3.0 mg/kg) followed two hours later by intravenous administration of xylazine hydrochloride (0.5 mg/kg). Passively emitted semen was collected into a funnel held beneath the stallion using a long pole. The funnel was lined with a disposable artificial vagina liner attached to a collection bottle fitted with a gel filter. Stallions were quietly observed for behavioral responses to treatment until either ejaculation occurred or until they recovered from sedation without ejaculation (out to approximately 70 minutes).

Traditional Semen Collection

Research stallions were allowed a minimum of 2 days rest after the chemical ejaculation attempt prior to traditional semen collection using an artificial vagina. To collect stallions with an artificial vagina, the Stallions were teased to a mare in estrus and then allowed to mount a breeding phantom. Semen was collected into a Colorado Model artificial vagina (Animal Reproduction Systems, Chino, CA) fitted with a collection bottle and disposable in-line nylon micromesh gel filter (Animal Reproduction Systems, Chino, CA).

Oxytocin Added Chemical Ejaculation

Stallions were allowed a minimum of 2 days rest prior to an attempt at pharmacologically induced ejaculation incorporating oxytocin into the protocol. The protocol was the same as for the traditional 'chemical ejaculation' protocol using the standardized dosages of imipramine hydrochloride and xylazine hydrochloride, with the addition of intravenous administration of oxytocin (20 units) immediately after administration of xylazine.

Prostaglandin Added Chemical Ejaculation

Stallions were allowed a minimum of 7 days rest prior to an attempt at pharmacologically induced ejaculation incorporating prostaglandins into the protocol. In this protocol stallions were administered the standard dose of imipramine hydrochloride followed two hours later by either: a) 250 μg of cloprostenol sodium intramuscularly followed five minutes later by 0.5 mg/kg xylazine intravenously (n=2) or b) administration of cloprostenol without xylazine (n=2).

Semen Evaluation

All pharmacologically induced and traditionally collected semen samples were evaluated for gel-free volume, sperm concentration, sperm motility and sperm morphology. Volume was measured in a warmed graduated cylinder. Sperm concentration was determined using a Densimeter 591B (Animal Reproduction Systems, Chino, CA) or a NucleoCounter® SP-100™ (ChemoMetec, Allerod, Denmark). Total and progressive sperm motility were evaluated by computer assisted semen analysis (SpermVision®, Minitube of America, Inc., Verona, WI) attached to a ZeissAX10 microscope. Sperm morphology was evaluated using a differential

interference contrast (DIC) microscope (OlympusEX51) at 1,000x magnification (10x eyepiece x 100x lens) and high viscosity microscope immersion oil (Resolve™, Thermo Scientific, USA).

Statistical Analysis

Semen parameters (volume, concentration, total sperm number, total motility and progressive motility) of chemical and standard collections were compared by ANOVA.

Differences in the percentage of stallions that ejaculated using the three protocols were compared by Chi Square. Differences in semen parameters (volume, concentration, total motility, progressive motility and percentage of morphologically normal sperm) were determined by ANOVA. Data are presented as the mean ± standard deviation. Values were considered statistically significant at $p<0.05$.

Results

Retrospective Rates Using Chemical Ejaculation

A total of 50 attempts at pharmacologically induced ejaculation were performed on a total of 12 stallions over the 6 year period (2013 to 2018). Semen was collected successfully on 22 (44 %) of the attempts. Four of the stallions presented with musculoskeletal issues, four presented ejaculatory dysfunction, two had neurological issues, one had squamous cell carcinoma lesions on his penis and one had a history of hemospermia. Four of the 12 clinical stallions never ejaculated in response to the chemical ejaculation protocol, but the 8 stallions ejaculated at least one time in all of their attempts. Only one stallion ejaculated in every chemical ejaculation attempt. As a comparison, semen parameters were recorded for 22

normal stallions collected using an artificial vagina at similar calendar dates to the successful chemically ejaculations.

Semen parameters from the 22 successful chemical ejaculations and 22 standard ejaculates are presented in Table 1. Total motility and progressive motility were the only semen parameters that were statistically different ($p<0.05$). Gel-free volume, sperm concentration, and total sperm numbers were not statistically different.

Table 1. Semen parameters for 22 clinical ejaculates collected using an imipramine-xylazine protocol and 22 stallions collected using an artificial vagina.

Parameter	Chemical Collection Mean ± SD (range)	Standard Collection Mean ± SD (range)	P-value
Interval from xylazine to emission of semen (minutes)	13.4 ± 10.6 (3 to 34)	N/A	N/A
Gel-Free Volume (mls)	37.7 ± 26.8 (8.2 to 130)	39.9 ± 20.6 (17 to 100)	0.765
Sperm Concentration ($x10^6$/ml)	443.3 ± 401.7 (141 to 873)	318.3 ± 282.3 (71 to 1270)	0.239
Total Sperm Number	12,512 ± 7,375 (3,240 to 33,150)	10,579 ± 7,718 (3,060 to 22,625)	0.400
Total Sperm Motility (%)	60.6 ± 22.3 (15 to 89)	74.1 ± 13.2 (46 to 92)	0.019
Progressive Sperm Motility (%)	52.8 ± 23.2 (10 to 84)	69.2 ± 14.5 (37 to 90)	0.007

Research Stallions - Ejaculation Rates

All seven stallions ejaculated when collected off a breeding phantom using an artificial vagina. The success rate of semen collection was significantly different ($p<0.05$) between the normal collection procedure (artificial vagina) and all chemical ejaculation procedures.

Oral administration of 3.0 mg/kg of imipramine hydrochloride followed 2 hours later by intravenous administration of 0.5 mg/kg of xylazine hydrochloride resulted in passive emission of semen in 3 of the 7 stallions (43 %) after only one attempt. The average time interval

between xylazine administration and ejaculation was 33.1 minutes (range 31 to 35 minutes). The ages of the stallions for which 'chemical ejaculation' was successful were 22, 22, and 27 years, whereas the ages of the stallions for which 'chemical ejaculation' was not successful were 17, 22, 22 and 27. None of the seven research stallions, that were treated with the tradition imipramine/xylazine protocol, ejaculated when 20 units of oxytocin was administered concurrently with xylazine hydrochloride. Stallions did exhibit a greater degree of spasmodic muscle contractions in the dorsal scrotal region when oxytocin was included in the protocol as compared to the original protocol.

None of the 4 research stallions, previously treated with the imipramine/xylazine protocol, ejaculated when a prostaglandin analogue was administered after imipramine. However when prostaglandins were added to the procedure, all of the stallions exhibited muscular contractions in the dorsal scrotal region, dropped their penis and gained an erection within 5 minutes after prostaglandin administration. Sweating and mild diarrhea was observed in 2 of the 4 stallions administered prostaglandins.

Research Stallions - Semen Parameters

There were no differences ($p>0.05$) between gel-free volume, sperm concentration, total sperm motility, progressive sperm motility, and total sperm numbers between ejaculates collected using chemical means from the three stallions using the imipramine-xylazine protocol and the normal ejaculates collected from all seven stallions, using an artificial vagina (Table 2). There were also no differences ($p>0.05$) in semen parameters when comparing ejaculates from

the same three stallions collected using the imipramine xylazine protocol or using an artificial vagina.

Table 2. Semen parameters for ejaculates collected using an imipramine-xylazine protocol (n=3) or using an artificial vagina (n=7).

Parameter	Imipramine-Xylazine	Artificial Vagina	p-Values
Gel-Free Volume (mls)	44 ± 40.7	41.1 ± 18.8	0.92
Sperm Concentration (x 10^6/ml)	795 ± 617.4	342.2 ± 253.4	0.26
Total Sperm Motility (%)	89.7 ± 2.1	66.9 ± 22.5	0.28
Progressive Sperm Motility (%)	85 ± 4.6	59.9 ± 23.8	0.29
Total Sperm Number (x 10^9)	19.7 ± 7.2	11.2 ± 8.9	0.97

Discussion

The success rate of chemical ejaculation in our clinical program (44 %) was similar to what has been reported previously (33 to 68 %) (McDonnell and Odian, 1994; Johnston and DeLuca, 1998; McDonnell, 2001). Success for individual stallions ranged from 100 % (i.e. 5 for 5 on one stallion) to 0 % (a combined 0 for 9 attempts for 4 stallions). The rational for the prospective part of the study was to determine if the addition of oxytocin or prostaglandins to the standard protocol would improve success of 'chemical ejaculation' in a population of aged stallions.

The standard imipramine-xylazine protocol success rate for aged stallions in the current study (43 %) was in the same range as in our clinical practice. McDonnell and Love (1991) noted that a specific titration dose modified for each individual stallion significantly improved the success rate of chemical ejaculation. Limited anecdotal data has been reported on the effects of oxytocin or prostaglandins on pharmacologically induced emission of semen in stallions (McDonnell, 1992).

Oxytocin is a peptide hormone synthesized in the paraventricular nuclei of the hypothalamus and secreted from the posterior pituitary gland. Oxytocin administration to stallions stimulates contraction of smooth muscles of the ductus deferens and epididymis and has been used clinically to alleviate blockage of plugged ampullae (McDonnell, 1992). It was hypothesized in the current study that oxytocin would enhance the success of imipramine-xylazine induced emission of semen by stimulation of smooth muscle contraction. However, none of the stallions ejaculated when oxytocin was added to the protocol.

Prostaglandin $F_{2\alpha}$ is a fatty acid hormone which can also induce smooth muscle contraction. A previous report noted that spontaneous ejaculations were observed within 10 minutes after intramuscular administration of an analogue of prostaglandin $F_{2\alpha}$ (McDonnell, 1992). In the current study, none of the stallions ejaculated when a single dose of prostaglandins was incorporated into the protocol. The type of prostaglandins selected, the dose administered and the timing of administration could all have affected the results.

Pharmacologically induced ejaculates are reported to be lower in volume and higher in sperm concentration in comparison to traditional ejaculates (McDonnell, 2001). However, in the present study, all semen parameters were similar between the seven traditional ejaculates and the three pharmacologically induced ejaculates in the aged research stallions.

Passive emission of semen with ‘chemical ejaculation’, if successful at all, usually occurs within 3 to 5 minutes after administration of xylazine as the stallion is becoming sedated or less commonly when the stallion is recovering from sedation (McDonnell and Odian, 1994; McCue and Ferris, 2018). In the present study, passive emission of semen occurred in all three stallions

approximately 30 minutes after administration of xylazine. None of the stallions emitted semen in the early period after administration of xylazine.

Summary

Pharmacologically induced ex copula ejaculation was successful in 44 % of 50 attempts in a clinical reproduction program and three of seven (43 %) of aged stallions using a standard protocol of imipramine and xylazine. Addition of oxytocin or cloprostenol to the standard protocol at the dosages and times used apparently had a deleterious effect on passive emission of semen as all attempts were unsuccessful.

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APPENDIX 2

MODIFIED TECHNIQUES

Immunohistochemistry Staining of Nerve Growth Factor-β

Day 1 of Immunostaining

Ensure all reagents needed for the experiment are diluted to the necessary dilution before beginning immunostaining each day. The prep work for day 1 includes:

- All slides labeled with appropriate tissue and date. Add additional labels if troubleshooting antigen retrieval methods, different antibodies, or different controls.
- Make new ethanol dilutions and set up deparaffinization gradient for every new experiment.
- Dilute 100x citrate buffer (pH 6.0) in deionized water to make 1x citrate buffer solution.
- Make fresh wash buffer solution for every new experiment. Dilute 25x TBS (tris buffered saline, pH 7.4; 64248; Abcam, Cambridge, United Kingdom) with Triton™ X in deionized water to make 1% TBS and 0.005% Triton™ X wash buffer solution. TBS was used over PBS to reduce background staining for the finished slides.
- Make new general blocking solution for every new experiment or run. Dilute normal goat serum (7481; Abcam, Cambridge, United Kingdom) to 5% in universal block solution made from bovine serum albumen. Any unused general blocking solution can be stored at 5°C.
- Bring hydrogen peroxide blocking reagent to room temperature or make more solution by diluting hydrogen peroxide to 0.05%.
- Dilute polyclonal rabbit antiserum raised against human NGF (PA5-14872; Thermo Fisher Scientific, Waltham, MA) in antibody diluent (64211; Abcam, Cambridge, United Kingdom). Vortex the raw antibody before diluting in the diluent. The antibody should be diluted 1:100. Past successful protocol used 1:50 dilution for the same antibody. Avoid freezing/thawing cycles for the raw antibody by thawing only the amount needed for the experiment. Approximately 300-500µl of diluted antibody will be needed. Do not bring the antibody or diluted antibody to room temperature.

Immnostaining will start with deparaffinization. All of the slides will be submerged in an ethanol gradient for 5 minutes at each step. Slides will need to be agitated halfway through each incubation to ensure proper deparaffinization. Steps include:

1. Xylene

2. Xylene
3. Xylene
4. 100% ethanol
5. 100% ethanol
6. 90% ethanol
7. 70% ethanol
8. 50% ethanol
9. Deionized water
10. Deionized water

Antigen retrieval will follow deparaffinization. Heat induced epitope retrieval (HIER) with 1x citrate buffer solution (pH 6.0) was determined to work the most efficient for this protocol. Slides were submerged in 1x citrate buffer solution within a pressure cooker for 3 minutes. Slides were then allowed to cool for 30 minutes on the counter to allow for full antigen retrieval. After cooling, the slides were washed twice for 5 minutes with deionized water.

Other antigen retrieval methods were used in a troubleshooting run including another HIER with Tris EDTA or proteinase induced epitope retrieval methods (PIER) using either proteinase A or trypsin. Citrate buffer yielded the best results.

After antigen retrieval, the slides go through a wash step. Slides are submerged in the wash buffer solution twice for 5 minutes along with agitation of the slides halfway through each incubation time.

Blocking steps follow the antigen retrieval steps. The first blocking solution applied to the slides is the general block. The slides will be submerged in the general block solution for at least 60 minutes at room temperature. A hydrophobic barrier is applied with a PAP pen (008899; Thermo Fisher Scientific, Waltham, MA) to each individual slide after the general block incubation is complete. The next blocking step is for hydrogen peroxide. 300-500 µl of hydrogen peroxide blocking solution will be applied to each slide. Allow slides to incubate for 15 minutes at room temperature. Wash hydrogen peroxide solution off of slides with wash buffer solution twice.

The final step for the first day of immunostaining is the application of the primary antibody. Vortex the antibody dilution before applying to the slides. The primary antibody should be distributed to every slide except for the negative controls. Add only antibody diluent to the negative controls. Let the slides incubate overnight (12-16 hours) at 4°C.

Day 2 of Immunostaining

Dilute and make all reagents needed for the second day of immunostaining. The prep work for day 2 includes:

- Create more wash buffer solution

- Dilute the secondary antibody (goat anti-rabbit IgG-HRP; G-21234; Thermo Fisher Scientific, Waltham, MA) to 1:500 in the same antibody diluent used for the primary.
- Prepare 3,3' diaminobenzidine (DAB) substrate kit (64211; Abcam, Cambridge, United Kingdom). Add 3µl (1 drop) of DAB chromogen to 1.5ml of DAB substrate. Allow mixture to get to room temperature.

After incubating overnight, the slides will go through a thorough wash step including 3 rinses with 1x TBS followed by soaking the slides 3 times with wash buffer solution for 5 minutes. The secondary antibody dilution will be applied to the slides next. Vortex antibody solution before applying to the slides. Incubate for at least 60 minutes at room temperature. After the incubation period, the slides will go through the same wash steps it did after the primary antibody.

The slides will then undergo colorimetric staining with the DAB kit and Hematoxylin solution (220365; Abcam, Cambridge, United Kingdom). The premade DAB mixture will be applied to the first for 10 minutes at room temperature. Wash the slides off with wash buffer 3 times then apply the hematoxylin solution. Incubate for at least 1 minute but do not let it sit too long. Wash the slides off with wash buffer 3 times. Remove the hydrophobic barrier with a xylene wash and coverslip the slides.

Immunohistochemistry Dilutions

Deparaffinization Gradient:

Reagent Needed	Combine	Result
100% Ethanol	200ml of 200 proof Ethanol	200ml of 100% Ethanol
95% Ethanol	190ml of 200 proof Ethanol 10ml of DI water	200ml of 95% Ethanol
70% Ethanol	140ml of 200 proof Ethanol 60ml of DI water	200ml of 70% Ethanol
50% Ethanol	100ml of 200 proof Ethanol 100ml of DI water	200ml of 50% Ethanol

Antigen Retrieval:

Retrieval Method	Reagent Needed	Combine	Result
HIER	1x Citrate Buffer	1000µl of 100x Citrate Buffer 99ml of DI water	100 ml of 1x Citrate Buffer solution
	1x Tris EDTA Buffer	1000µl of 100x Citrate Buffer 99ml of DI water	100ml of 1x Tris EDTA Buffer solution
PIER	1:1 Trypsin: Buffer	500µl of Trypsin concentrate 500µl of Buffer (TBS or PBS)	1ml of 1:1 Trypsin Buffer solution
	TBS Wash Buffer	25ml of 20x TBS 475ml of DI water	500ml of 1x TBS solution
	PBS Wash Buffer	100ml of 10x PBS 900ml of DI water 500µl of tween	1000ml of 1x PBS 0.05% tween wash buffer
	5% Normal Goat Serum Blocking Solution	50ml Normal Goat Serum 950ml Universal Block Solution	1000ml of Normal Goat Serum blocking solution

Primary Antibody:

Antibody	Combine	Result	Dilution
Mouse Monoclonal anti-NGF	5µl of Mouse Monoclonal 995µl of Antibody Diluent	1ml of diluted antibody	1:200
Rabbit Polyclonal anti-NGF	10µl of Rabbit Polyclonal 990µl of Antibody Diluent	1ml of diluted antibody	1:100

Secondary Antibodies:

Antibody	Combine	Result	Dilution
Goat anti-Mouse	3µl of Goat anti-mouse 1497µl of Antibody Diluent	1.5ml of Diluted Antibody	1:500
Goat anti-Rabbit	3µl of Goat anti-Rabbit 1497µl of Antibody Diluent	1.5ml of Diluted Antibody	1:500