



An immunohistochemical study of ovarian innervation in the emu (*Dromaius novaehollandiae*)

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ABSTRACT

MADEKUROZWA, M-C. 2008. An immunohistochemical study of ovarian innervation in the emu (*Dromaius novaehollandiae*). *Onderstepoort Journal of Veterinary Research*, 75:59–65

The present study investigated the distribution of nerves in the ovary of the emu. The neuronal markers, protein gene product 9.5, neurofilament protein and neuron specific enolase demonstrated the constituents of the extrinsic and intrinsic ovarian neural systems. The extrinsic neural system was composed of ganglia in the ovarian stalk, as well as nerve bundles, which were distributed throughout the ovary. Isolated neuronal cell bodies, in the medulla and cortex, formed the intrinsic neural system. An interesting finding of the study was the presence of nerve bundles, circumscribed by lymphocytes, in the ovarian stalk. The findings of the study indicate that the distribution of nerve fibres and neuronal cell bodies in the emu ovary is similar, but not identical to that of the domestic fowl and ostrich.

Keywords: Emu, immunohistochemistry, innervation, ovary

INTRODUCTION

Extensive research has been conducted on the innervation of the ovary in both birds (Gilbert 1965; 1968; 1969; Unsicker, Seidel, Hofmann, Muller, Schmidt & Wilson 1983) and mammals (Lakomy, Doboszynska & Sztejn 1983; Dees, Hiney, McArthur, Johnson, Dissen & Ojeda 2006). Early reports described the ovary as being supplied by predominantly sympathetic nerves, which form a component of the ovarian nerve plexus (Gilbert 1965; 1968; 1969). These sympathetic nerves form part of a neuronal pathway through which the central nervous system exerts a regulatory influence on the ovary (Gerendai, Kocsis & Halasz 2002).

The sympathetic nerves supplying the ovary are generally adrenergic in nature. These extrinsic adrenergic nerves have a myriad of functions, which include

the regulation of blood flow (Lakomy *et al.* 1983), folliculogenesis (Curry, Lawrence & Burden 1984; Kannisto, Owman, Rosengren & Walles 1984), and ovarian steroidogenesis (Weiss, Dail & Ratner 1982; Sporrang, Kannisto, Owman, Sjoberg & Walles 1985; Aguado 2002; Delgado, Sosa, Dominguez, Casais, Aguado & Rastrilla 2004). In addition to the extrinsic sympathetic nerves supplying the ovary, an intrinsic neuronal system has also been identified. The intrinsic innervation of the ovary is composed of neuronal cell bodies, which are generally catecholaminergic (D'Albora & Barcia 1996; D'Albora, Lombide & Ojeda 2000; Anesetti, Lombide, D'Albora & Ojeda 2001). Although the exact role of ovarian neuronal cell bodies is unclear, they are thought to be involved in the inhibition of steroidogenesis and in the regulation of ovarian blood flow (D'Albora, Anesetti, Lombide, Dees & Ojeda 2002).

It is clear from the literature that both the extrinsic and intrinsic ovarian innervation is vital to the normal functioning of the ovary. Indeed it has been

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suggested that ovarian pathologies may result from a dysfunction in innervation (Anesetti *et al.* 2001; Lara, Dorfman, Venegas, Luza, Luna, Mayerhofer, Guimaraes, Rosa E Silva & Ramirez 2002). To date much of the research conducted on the distribution and function of ovarian nerves has focused on mammals (Weiss *et al.* 1982; Lakomy *et al.* 1983; Curry *et al.* 1984; Dees, Hiney, Schulte, Mayerhofer, Danilchik, Dissen & Ojeda 1995; Dees *et al.* 2006). Furthermore, apart from a recent study conducted on the ovary of the immature ostrich (Kimaro & Madekurozwa 2006), most of the research carried out on birds has been limited to the domestic fowl (Gilbert 1965; 1968; 1969; Unsicker *et al.* 1983). Although the emu is an economically important ratite there is currently a lack of information on the ovarian innervation of this avian species. The present study describes the distribution of nerve fibres in the ovary of the emu using antibodies against neurofilament protein (NP), protein gene product 9.5 (PGP 9.5) and neuron specific enolase (NSE). Neurofilament protein is expressed specifically in neurons (Ohara, Gahara, Miyake, Teraoka & Kitamura 1993), while PGP 9.5 and NSE are cytoplasmic markers of both neuronal and neuroendocrine cells (Martin, Fraile, Peinado, Arenas, Elices, Alonso, Paniagua, Martin & Santamaria 2000; Theodoropoulos, Tsigka, Mihalopoulou, Tsoukala, Lazaris, Patsouris & Ghikonti 2005).

MATERIALS AND METHODS

A total of eleven female emus aged 18 months and weighing 35–40 kg were used in the present study. The birds originated from a commercial farm in the North West Province of South Africa. All the birds had active ovaries, which contained approximately 30 predominantly yellow-yolk follicles, with the diameters of the largest follicles ranging from 20 to 30 mm. The birds were sampled in March. The emu being a short day breeder typically begins to lay in April in the southern hemisphere (Minnaar & Minnaar 1997).

The birds were slaughtered at a commercial abattoir, employing a standard slaughter protocol. Ovarian tissue samples were obtained from the birds 10–15 min after slaughter. The tissue samples were then immersion-fixed in either 4% paraformaldehyde (pH 7.2) or Bouin's fluid for 12 h. Some samples were fixed in Bouin's fluid because the antibody against PGP 9.5 reacts better with antigens in paraffin sections. The samples fixed in paraformaldehyde were then placed for 24 h at 4 °C, in a 30%

sucrose solution made up in 0.01 M phosphate buffered saline solution (PBS, pH 7.4). Thereafter, they were snap-frozen in OCT compound (Sakura, CA, USA) in isopentane slurry and then stored at –80 °C. Tissue samples fixed in Bouin's fluid were processed routinely for histology and embedded in paraffin wax.

The immunostaining technique was performed on 10 µm-thick cryostat sections and 5 µm-thick paraffin sections, using a LSAB-plus kit (Dakocytomation, Denmark). The cryostat sections were air-dried for 60 min at room temperature after which they were rinsed in PBS. Endogenous peroxidase activity in both cryostat and paraffin sections was blocked, using a 3% (v/v) hydrogen peroxide solution in water for 5 min. The slides were then rinsed in PBS for 5 min. Thereafter, the paraffin sections were microwaved at 750 W for two cycles of 7 min each, and after being allowed to cool for 20 min, they were rinsed with PBS and then incubated for 60 min at room temperature in a solution containing a polyclonal antibody against PGP 9.5, at a dilution of 1:50. The cryostat sections were incubated for 30 min in a solution containing monoclonal antibodies against NP, at a dilution of 1:25, and a ready-to-use solution of antibodies against NSE, for 60 min. After this incubation all tissue sections were rinsed with PBS and then incubated for 15 min in a solution containing a biotinylated secondary antibody (LSAB-plus kit, Dakocytomation, Denmark). Thereafter, the slides were rinsed in PBS and subsequently incubated for 15 min with the streptavidin peroxidase component of the LSAB-plus staining kit. Tissue sections were then rinsed in PBS and bound antibody was visualized after the addition of a 3,3'-diaminobenzidine tetrachloride solution (LSAB-plus kit, Dakocytomation, Denmark). Tissue sections were counterstained with Mayer's haematoxylin before being dehydrated in graded concentrations of ethanol.

In the negative controls the primary antibodies were replaced with either normal mouse or rabbit serum. A histological section of brain tissue was used as a positive control.

RESULTS

The ovary was composed of an ovarian stalk, medulla and cortex. Numerous nerve bundles immunopositive for NSE, NP and PGP 9.5 coursed through the ovarian stalk and medulla (Fig. 1). Some of the nerve bundles were associated with blood vessels. In addition, a few nerve bundles in the ovarian stalk were closely associated with lymphoid follicles (Fig.

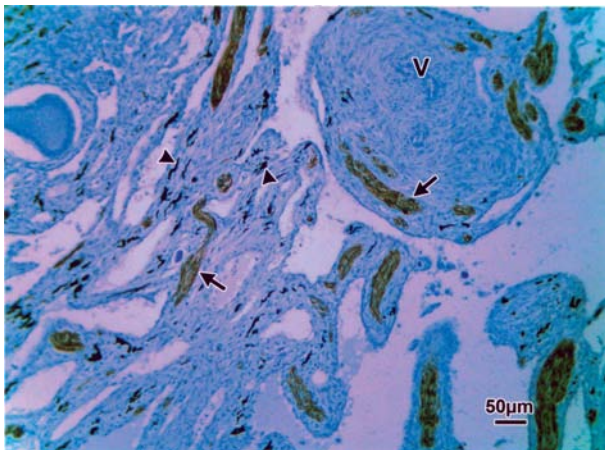


FIG. 1 NP immunoreactive nerve bundles (arrows) in the medulla. Some of the nerve bundles are associated with blood vessels (V). Arrowheads: melanocytes

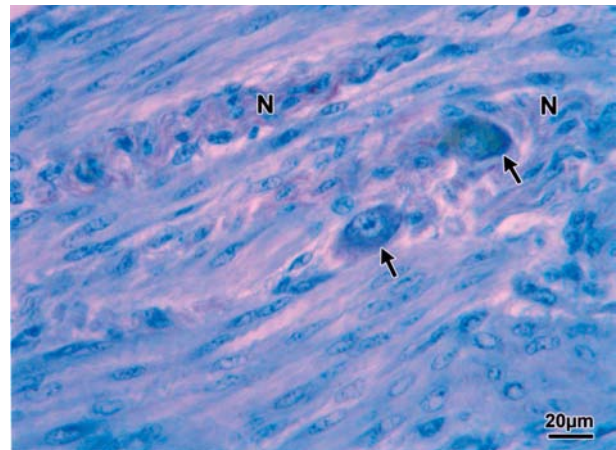


FIG. 4 NSE immunoreactive neuronal cell bodies (arrows) in the medulla. N: nerve bundle

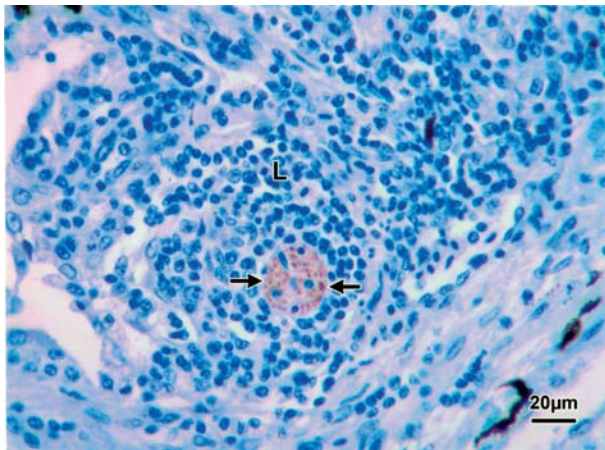


FIG. 2 NP immunoreactive nerve bundle (arrows) surrounded by lymphocytes (L)

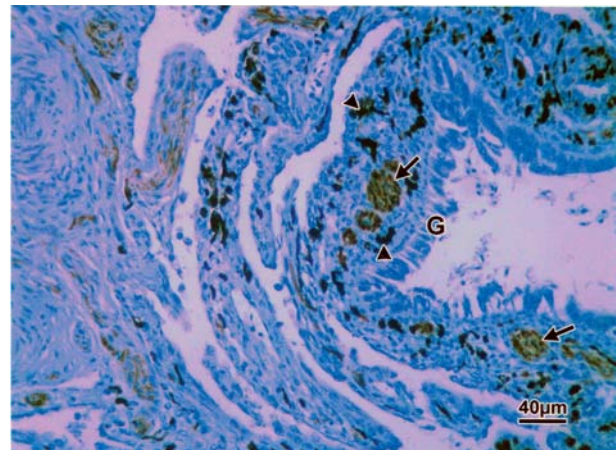


FIG. 5 PGP 9.5 immunopositive nerve fibres (arrows) in the upper limits of the cortex. Arrowheads: melanocytes. G: germinal epithelium

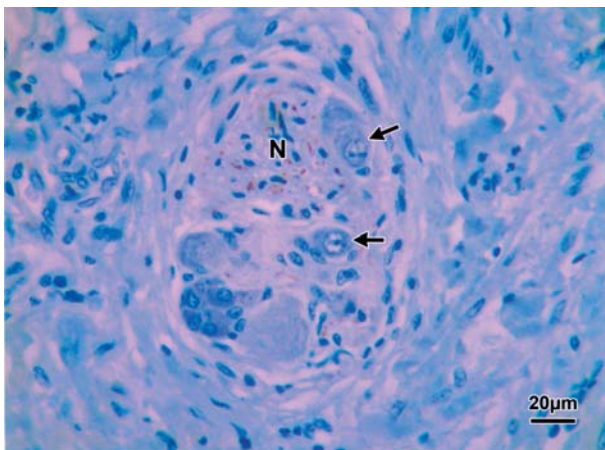


FIG. 3 NP immunonegative neuronal cell bodies (arrows) form a ganglion in the ovarian stalk. Closely associated with the neuronal cell bodies is a NP immunopositive nerve bundle (N)

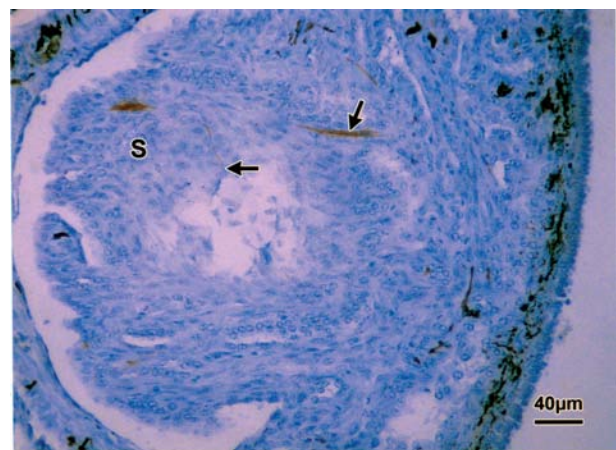


FIG. 6 Infiltration of an atretic previtellogenic follicle by connective tissue stroma (S) and NP immunoreactive nerve fibres (arrows)

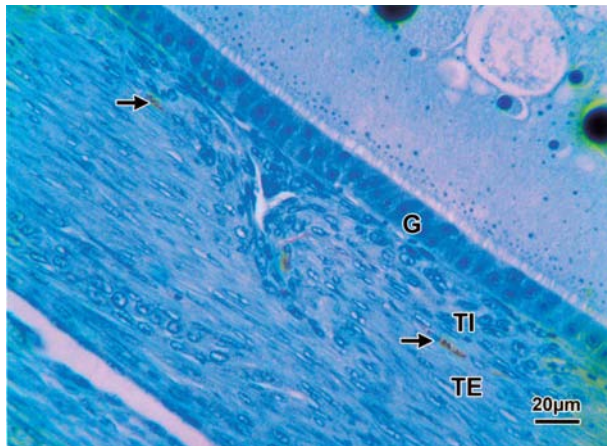


FIG. 7 PGP 9.5 immunopositive nerves (arrows) in the theca externa (TE) of a developing vitellogenic follicle. TI: theca interna. G: granulosa cell layer

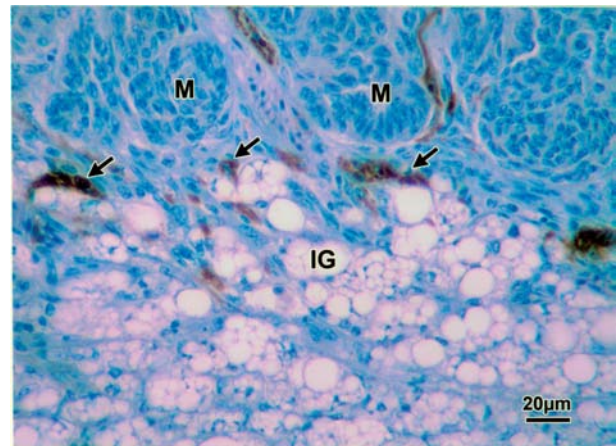


FIG. 8 A network of NSE immunoreactive nerve fibres (arrows) is present at the interface between intramural veins (M) and interstitial gland cells (IG) in an atretic vitellogenic follicle

2). Ganglia, composed of clusters of neuronal cell bodies were present in the ovarian stalk (Fig. 3). The neuronal cell bodies were characterized by the presence of a large round nucleus with a prominent nucleolus. The cytoplasm of these cell bodies was immunopositive for NSE and PGP 9.5, but immunonegative for NP. Two to three layers of fibrocytes circumscribed the ganglia. Isolated neuronal cell bodies were demonstrated in the medulla. In some instances the neuronal cell bodies were associated with nerve bundles (Fig. 4).

The cortex contained primordial, pre-vitellogenic and vitellogenic follicles. Nerve bundles in the cortex were not closely associated with developing primordial and previtellogenic follicles, but rather occurred in the connective tissue stroma between the follicles. These cortical nerve bundles branched to form a neural network in the superficial regions of the cortex (Fig. 5). Although nerve fibres were not seen around developing previtellogenic follicles, they were observed in atretic previtellogenic follicles during the infiltration of the follicle by connective tissue (Fig. 6). In addition to nerve fibres occasional neuronal cell bodies were observed in the cortex.

Vitellogenic follicles were suspended by follicular stalks, which were extensions of the cortex. Several nerve bundles coursed through the follicular stalks and branched to form perifollicular nerve fibres in the superficial tunic and theca externa of the developing vitellogenic follicles (Fig. 7). Most of the perifollicular nerve fibres were associated with blood vessels.

The early stages of atresia in vitellogenic follicles were characterized by the proliferation of theca in-

terna and granulosa cells. A neural network was present in the region between the theca interna and granulosa cell layer. The theca interna and granulosa cells subsequently differentiated into interstitial gland cells, which infiltrated both the theca externa and oocyte. Eventually the interstitial gland cells abutted intramural veins, which were located in the outer limits of the theca externa. At this stage of atresia a dense network of nerve fibres was observed between the interstitial gland cells and intramural veins (Fig. 8). The interstitial gland cells eventually dispersed to form stromal interstitial gland cells, which were located in the superficial regions of the cortex. Nerve fibres were observed coursing between the stromal interstitial gland cells.

DISCUSSION

The neuronal markers used in the study have clearly demonstrated the components of both the extrinsic and intrinsic neural systems of the emu ovary. The extrinsic system was composed of ganglia in the ovarian stalk and nerve bundles in the ovarian stalk, medulla, cortex and follicular stalks. Isolated neuronal cell bodies in the medulla and cortex formed the intrinsic neural system.

As in the domestic fowl (Gilbert 1965; 1968; 1969), extrinsic nerve bundles entered the ovary through the ovarian stalk where they subsequently formed a nerve plexus. The ovarian nerve plexus in both the emu and domestic fowl (Gilbert 1965; 1968; 1969; Unsicker *et al.* 1983) contained several encapsulated ganglia. In the present study the nerves forming the plexus were immunopositive for NSE, NP

and PGP 9.5, while the ganglia were immunopositive for NSE and PGP 9.5, but immunonegative for NP. In the emu, ostrich (Kimaro & Madekurozwa 2006) and domestic fowl (Gilbert 1965; 1968) nerves in the ovarian stalk coursed into the medulla, cortex and follicular stalks.

The role of nerves in blood regulation is well established (Lakomy *et al.* 1983). Indeed, the close association between nerves and blood vessels has been reported in the ovaries of the rat (Lawrence & Burden 1980), domestic fowl (Unsicker *et al.* 1983), and pig (Lakomy *et al.* 1983). In the present study, perivascular nerve fibres were demonstrated throughout the ovary. Admittedly, the antibodies used precluded the identification of adrenergic and cholinergic nerves. Both cholinergic and adrenergic nerves have been demonstrated in the domestic fowl (Gilbert 1969). However, it was conceded that most of the nerves were adrenergic. This is supported by research conducted in the rat in which most perivascular nerves are adrenergic (Lawrence & Burden 1980). Although the association between nerves and blood vessels has been extensively reported, the relationship between nerves and lymphoid follicles has received far less attention. An interesting finding of the present study was the close association between nerves and lymphoid follicles in the ovarian stalk. Research conducted by Felten, Ackerman, Wiegand & Felten (1987) on the spleen suggests that neurotransmitters released from nerve endings regulate the migration, maturation, proliferation and activation of lymphocytes. The relevance of the close association of nerve bundles and lymphoid follicles in the emu ovary is unclear.

The distribution of nerve fibres in the medulla, cortex, follicular stalks and perifollicular regions of birds appears to be fairly uniform (Gilbert 1968; 1969; Kimaro & Madekurozwa 2006). Indeed the distribution pattern of perifollicular nerve fibres in the developing follicles of the emu varied little from that of the ostrich (Kimaro & Madekurozwa 2006). In both raptorial birds nerves formed a network in the thecal layer. No nerve fibres were seen within the granulosa cell layer. The presence of a thecal neural network and the lack of nerve fibres within the granulosa are consistent with observations made in the domestic fowl (Gilbert 1965; 1969). It is thought that in mammals perifollicular nerve fibres play a role in folliculogenesis (Curry *et al.* 1984; Kannisto *et al.* 1984; Malamed, Gibney & Ojeda 1992) and ovulation (Bahr, Kao & Nalbandov 1974; Luna, Cortes, Flores, Hernandez, Trujillo & Dominguez 2003). In addition, based on the findings of the present study, it is prob-

able that perifollicular nerves are involved in the differentiation of thecal and granulosa cells into interstitial gland cells during atresia. This is evidenced by the presence of numerous nerve fibres in regions of interstitial gland formation in atretic vitellogenic follicles. The presence of a neural network in contact with differentiating interstitial gland cells is not surprising, as it has been shown that nerves have a profound effect on the development, maintenance and function of corpora lutea in the bovine (Kotwica, Bogacki & Rekawiecki 2002), pig (Jana, Dzień, Panczyszyn, Rogozinska, Wojtkiewicz, Skobowiat & Majewski 2005) and rat (Casais, Delgado, Sosa & Rastrilla 2006).

In addition to the extrinsic neural system described above, the ovaries of birds and mammals contain an intrinsic neural system. The exact function of the intrinsic neural system is unclear. However, it is thought that this neural system is concerned not only with follicular maturation and blood regulation, but also with the inhibition of steroidogenesis (D'Albora *et al.* 2002). The intrinsic neural system is composed of isolated neuronal cell bodies. As in the domestic fowl (Gilbert 1965; 1968), the neuronal cell bodies in the emu were characterized by the presence of a round nucleus with a prominent nucleolus. Significantly the results of the present study not only confirm the findings of Gilbert (1965; 1968), with regard to the morphology of neuronal cell bodies, but also further characterize the physiological nature of these cells. The fact that the neuronal cell bodies were immunopositive for both NSE and PGP suggests that they possess a neuroendocrine function. This is supported by research on the rat ovary in which neuronal cell bodies have been shown to produce catecholamines as evidenced by the presence of tyrosine hydroxylase, an enzyme involved in the synthesis of norepinephrine.

The distribution and number of neuronal cell bodies appear to be species specific. In the emu a few neuronal cell bodies were demonstrated in the medulla and occasionally in the cortex. Likewise, in the ostrich a few neuronal cell bodies were observed in the medulla (Kimaro & Madekurozwa 2006). In contrast, numerous neuronal cell bodies were observed in the follicular stalks supporting mature follicles in the domestic fowl (Gilbert 1965). In this species very few neuronal cell bodies were observed in the cortex or in association with developing follicles (Gilbert 1969). Furthermore no neuronal cell bodies were observed in the medulla. In both the immature ostrich (Kimaro & Madekurozwa 2006) and the emu neuronal cell bodies were occasionally associated

with nerve bundles. Similar observations have been made in the human ovary where it is thought that the association of neuronal cell bodies with nerve bundles implies a regulatory association between the intrinsic and extrinsic neural systems of the ovary (Anesetti *et al.* 2001).

In conclusion, this study has described the salient features of the extrinsic and intrinsic neural systems of the emu ovary. The distribution of nerve fibres in the emu ovary is similar to that of the domestic fowl (Gilbert 1965; 1968; 1969) and ostrich (Kimaro & Madekurozwa 2006). A distinguishing feature of ovarian innervation in the emu is the close association between nerve bundles and lymphoid follicles in the ovarian stalk. Further research on the significance of this finding needs to be conducted.

ACKNOWLEDGEMENTS

I thank the technical staff in the University of Pretoria's Department of Pathology and Department of Education Innovation (Creative Studios) for their assistance. The University of Pretoria and the National Research Foundation (Thuthuka programme) funded this study.

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