Research Article

Nerve and Blood Vessel Wiring in Endometriotic Peritoneal Lesions

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ABSTRACT

Detection of unique nerve fibres in the endometrium of women with endometriosis and subsequently in their peritoneal lesions has led to increased interest in studying their relationship with infertility and pain. Blood vessels and nerves fibres course throughout the body in an orderly pattern, often alongside one another. Although superficially distinct, the mechanisms involved in wiring neural and vascular networks seem to share similarities. We found that nerve fibres and blood vessels traverse the endometriotic peritoneal tissue in slightly different ways with variations in length density (L_v), branch point density (B_v), segment length between branch points (L_v/B_v) and capillary radial diffusion distance r(diff). The capillary radial diffusion distance r(diff) and the spatial co-localisation distance in conjunction with nerve fibres fell within range for the facilitation of the physiological diffusion and transfer of molecular substances and the transmission of electrical signals to co-ordinate tasks within the endometriotic peritoneal lesion. Biological systems exist and operate in a three-dimensional environment and blood vessels and nerve trunks often travel in close apposition through many tissues; therefore, it was a priority to assess ($CD31^+$) blood vessel and ($PGP9.5^+$) nerve fibre three-dimensional structural features from qualitative and quantitative perspectives in peritoneal endometriotic lesions. Utilising laser scanning confocal microscopy allowed for considerable precision in the study of endometriotic peritoneal lesion blood vessel and nerve fibre co-localisation and three-dimensional relationships.

Keywords: Peritoneal, Endometriotic, Lesion, Blood vessels, Nerve fibre, Stereology

INTRODUCTION

Endometriosis appears to affect up to 20% of women through their reproductive lives [1]. However, the true prevalence of endometriosis is possibly higher, as some women with the condition experience no symptoms whilst others present with a great variety of symptoms [2,3]. Endometriosis is a benign gynecological condition and despite its relative frequent occurrence, its mechanisms still remain unclear. The condition is defined by the presence of endometrial-like tissue outside the uterine cavity.

Chronic pelvic pain is very common in women with endometriosis. Laufer et al. [4] reported that approximately 70% of women with pelvic pain had subsequent endometriosis confirmed by laparoscopy. Currently, a definitive diagnosis of endometriosis is made by visual inspection during surgery of the abdomen and pelvis with histological confirmation of biopsied lesions [5]. Laparoscopy is the most common surgical procedure used to inspect the peritoneal cavity [5,6].

Tulandi et al. (2001) was the first study that investigated the presence of the nerve fibres in peritoneal endometriotic lesions compared to normal peritoneum in women with endometriosis using an antibody against neurofilament (NF) protein. However, no differences in the density of nerve fibres in peritoneal endometriotic lesions compared to normal peritoneum were detected.

In a pioneering study Tokushige et al. [7] applied the pan-neuronal marker protein gene product 9.5 (PGP9.5) to the endometrium of 35 women with confirmed endometriosis, and identified nerve fibres in the endometrial functional layer, although none were found in the 82 women without endometriosis. Ninety percent of women with endometriosis, were observed to have nerve fibres in their endometrium Bokor et al [8]. Further, Tokushige et al. [9] observed significantly higher densities of small unmyelinated nerve fibres in the peritoneal endometriotic lesions of women with endometriosis compared with normal peritoneum in women without endometriosis. In addition, the authors observed that the nerve fibres were more frequently seen near endometriotic glands and blood vessels compared to the stroma.

Mechsner et al. (2007) confirmed these results by finding nerve fibres stained with NF and Substance P (SP) in direct contact with endometriotic lesions in 74.5% (79/106) of the

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samples. However, there were no significant differences in the total mean nerve scores in the peritoneum tissue from women with and without the disease. Mechsner et al. (2009) reported that the density of nerve fibres stained with NF and PGP9.5 was significantly higher in the peritoneum of women with higher pain scores for dysmenorrhoea and pelvic pain in comparison with the peritoneum from women with a lower pain score. However, there was no correlation between the nerve fibre density and dyspareunia, dyschezia or dysuria. In addition, some studies have also investigated the effect of hormonal treatment on nerve fibre density. Tokushige et al. (2009) reported a decrease in the density of nerve fibers stained with PGP9.5 in peritoneal endometriotic lesions of hormone-treated women in comparison to endometriotic lesion of untreated women. However, Wang et al. (2011) observed no differences in the density of nerve fibers stained with NF or PGP9.5 through the different stages of the menstrual cycle.

Maharajaa et al. (2014) reported increased neurogenesis in the stromal region in peritoneal lesions of endometriosis. The innervation of lesions correlated to the intensity of menstrual pain. It was speculated that nerve growth factor (NGF) in glandular epithelium may promote the growth of nerve fibres into the core of the lesions; however, the inverse correlation between NGF expression in glandular epithelium and menstrual pain were indicative that the mechanisms of pain generation in endometriosis were complex.

A better understanding of endometriotic lesion relationships between innervations and specific clinical characteristics may elucidate aspects of pain mechanisms and infertility in endometriosis and aid in the facilitation of the development of novel therapeutic approaches Maharajaa (2019).

Angiogenesis is the development of new blood vessels from pre-existing vessels and is a crucial force for shaping the nervous system and protecting it from disease [10]. Blood vessel formation is a major hallmark in the pathogenesis of endometriosis. The vascularization of endometriotic lesions is complex and involves angiogenesis, vasculogenesis and inosculation [11].

Blood vessels and nerves are vital channels to and from tissues and genetic insights have demonstrated that they have much more in common than was originally anticipated [12]. Blood vessels and nerve fibres course throughout the body in an orderly pattern, often alongside one another. Although superficially distinct, the mechanisms involved in wiring neural and vascular networks seem to share some deep similarities [13].

The evolution of multicellular organisms has allowed the development of specialised tissues to perform complex tasks. There are at least two key developmental stages which were crucial for vertebrates to achieve this goal. Firstly, the formation of a vascular system in which blood-vessel networks branch frequently to ensure that all tissues receive an adequate blood supply, and secondly, the development of a nervous system in which highly branched nerve networks transmit electrical signals to and from peripheral organs to coordinate tasks. Both these networks are laid down in a complex and ordered manner, which is controlled by developmental cues that ensure that they match the architectural and functional demands of specific tissues [12].

The peritoneal fluid of patients with endometriosis is a complex suspension carrying inflammatory cytokines, growth factors, steroid hormones, proangiogenic factors, macrophages, and endometrial and red blood cells. These cells and their signalling products concur to promote the spreading of new blood vessels at the endometriotic lesions and surroundings, which contributes to the endometriotic implant survival. Angiogenesis represents a critical step in the establishment and pathogenesis of endometriosis, this process has been viewed as a potential new target for therapeutic intervention [14].

It is a long and established belief that nerves and blood vessels usually travel together and in the most direct route towards their targets. Nerves are tougher than most other structures, followed by arteries, veins, and lymphatic vessels. Nerves should therefore follow the most direct route, and be followed by the arteries, veins, and lymphatic vessels [15]. Once a few nerve fibres have traversed a region, many other nerve fibres will follow along these to form a nerve bundle. The path of the nerve bundle will be reasonably straight in embryonic tissues, although obstacles such as blood vessels and supporting elements can cause temporary or permanent deviations [16]. It is suspected that these nerve "fibres" are neurites, which have developed from pre-exiting neurones and nerve trunks elsewhere in the pelvis [17].

A stereological method for estimating the length of anisotropic features, such as blood vessels, from datasets collected via laser scanning confocal microscopy (LSCM), was previously developed [18]. Using a stereological approach, it was possible to determine endometriotic peritoneal lesion length densities (total lengths per given volume of tissue or L_v) of blood vessels and nerve fibres. The branch point density (number per volume or B_v) of blood vessel and nerve fibre junctions. From these two measurements, it was possible to calculate the segment length between branch points of the blood vessels and nerve fibres (L_v/B_v) [19-21] in endometrial peritoneal lesions. From the L_v the capillary radial diffusion distance (facilitation of the passive transportation of metabolites) could also be determined for the blood vessels [19,22] in endometrial peritoneal lesions. The use of LSCM datasets also allowed for the determination of the spatial co-localisation distance between blood vessels and nerve fibres.

MATERIALS AND METHODS

Tissue collection

Archival tissue samples of peritoneal ectopic lesions (n=20; mean 37; range 20-45 years), from women with endometriosis were collected from the Tissue Pathology and Diagnostic Oncology, The Royal Prince Alfred Hospital (RPAH), Sydney, NSW, Australia. Samples were collected from women with endometriosis who had presented with pain symptoms and/or infertility; however, none had received any hormonal treatment within three months prior to tissue sampling, no preinvasive cervical disease, no local history of radiotherapy, fibroids, adenomyosis and cancer.

This study was approved by the Human Ethics Committees of the Sydney Local Health District (Royal Prince Alfred Hospital) and the University of Sydney, NSW, Australia (Protocol No X 14-0232 & HREC/10/RPAM/24.

Tissue samples had previously been fixed in 10% neutral buffered formalin for 18-24 hrs then processed and embedded in paraffin according to a standard protocol.

Micrometry

Initially the peritoneal lesions were sectioned at 4 μm and mounted onto glass slides (Superfrost Ultra Plus, Menzel Glaser, Braunschweig, Germany) and dried at 60ºC for 1 hr and routinely stained with Haematoxylin and Eosin (H&E) assessment, another section was immunohistochemically stained [23] for the detection of nerve fibres. Once confirmed the peritoneal lesion was further sectioned at 60μm and collected into a 15ml glass vial, in preparation for deparaffinisation in xylene and rehydrated via descending concentrations of alcohol (100-70%) to water.

Heat-induced epitope retrieval

Tris-EDTA buffer pH 8.0 antigen retrieval solution was prepared according to a published recipe [24]. The peritoneal sections were then treated with the retrieval solution in a 60°C water bath for between 18-24 hrs. Post retrieval the sections were allowed to cool at room temperature for 2 hrs.

Pre-treatment

Reducing auto-fluorescence

To reduce auto-fluorescence [25], a 0.001 gm/ml solution of sodium borohydride in Phosphate Buffered Saline (PBS) was prepared. The solution was applied immediately to the peritoneal lesion section, incubated in 6 changes for 10 mins each, then rinsed in physiological saline 4 changes for 5 mins to remove traces of the sodium borohydride. A final wash in Tris Buffered Saline containing, 0.3% Triton X-100 (TBS+0.3% Triton X-100) and a gentle agitation was applied for 2 changes of 5 min duration.

Enzyme pre-treatment

All peritoneal whole mount sections underwent an enzyme pre-treatment step with Collagenase Type II C6885-1G (500 U/ml) (0.0009 gm/ml) in PBS, incubated at 37°C for 10 mins and washed in TBS+0.3% Triton X-100 with a gentle agitation applied for 2 changes of 5 mins duration.

Immunofluorescence

Block

The following sequence was applied to the peritoneal sections. Non-specific (background) staining was reduced by pre-incubating for 24 hrs at 4°C with 10% normal goat serum, 2% BSA in Tris Buffered Saline. The slides were drained (not rinsed).

Antibodies

CD31 is expressed on all continuous endothelia, including those of arteries, arterioles, venules, veins and non-sinusoidal capillaries [26]. Whereas CD34 appears to be expressed at its highest level on the earliest progenitors and to decrease progressively with maturation [27]. Similarly both antibodies require heat-induced epitope retrieval, however, CD34 produced an abundance of background staining in our preliminary staining.

PGP9.5 antibody labels the neuronal cell bodies and axons in central and peripheral neural systems. A highly specific pan-neural marker which was used to detect all types of nerve fibres [7].

Staining

Staining was carried out with Monoclonal mouse anti-human CD31, Endothelial cell clone JC70A (M0823, DAKO, Corporation, Carpinteria, USA) and Polyclonal rabbit anti-Protein Gene Product 9.5 (PGP9.5) (Z5116, DAKO, Corporation, Carpinteria, USA). These primary antibodies were cocktailed and diluted 1:1200 and incubated at 4°C overnight. They were washed in TBS+0.3% Triton X-100 and gentle agitation was applied for 4 changes of 5 min duration.

The following secondary antibodies were cocktailed and diluted 1:1200 and incubated at 4°C overnight (Fluorescein labelled goat anti-mouse (FITC)) immunoglobulins (F-2761, Molecular Probes, Thermo Fisher Scientific, Waltham, Massachusetts, (USA). Tetramethylrhodamine labelled goat anti-rabbit (TRITC) immunoglobulins (T-2769, Molecular Probes, Thermo Fisher Scientific, Waltham, Massachusetts, (USA). They were washed in Tris Buffered Saline TBS+0.3% Triton X-100 and gentle agitation was applied for 4 changes of 5 min duration.

Laser scanning confocal microscopy (LSCM)

We have developed laser scanning confocal microscopy through a series of stages [18,24,28], where it can now be applied to precisely examine the three-dimensional relationships between blood vessels and nerve fibre structures within human peritoneal lesions.

A total of 2-4 confocal volumetric fields of view, ranging between 50-60 μm were visualised in peritoneal lesions. Thus, samples from 19 subjects generated a total of 57 confocal volumetric fields of view. Blood vessel and nerve fibre structures were visualised using immunofluorescence, Leica SP5 multiphoton microscope (Leica Microsystems, Pty, Ltd, Wetzlar, Germany) and specimens were illuminated with 488 (30%) and 561 (15%) laser power. Using the ability of confocal microscopy to optically section intact or thick specimens, sequential confocal fluorescence images of approximately 0.3 m optical section thickness were collected with the x25/N.A. 0.95 water objective through the full depth of each 50 μm section.

Pre-processing

The pre-processing step typically involved the application of image filters (mathematical algorithms implemented within the software) to the complete dataset to remove noise and artifacts, smooth or sharpen the images, or to correct problems such as contrast and/or brightness. Whilst these filters are normally applied as pre-processing sets, they could also be carried out after a three-dimensional model had been reconstructed from the image dataset.

Image enhancement

The application of a median and/or a Gaussian filter had the general effect of smoothing images; this was used to eliminate noise and background artefacts and to smooth sharp edges. By employing a sharpening filter to emphasize details in the image dataset, was most useful when the image dataset consisted of fine structural components such as those found in endometriotic peritoneal lesions, or when edge enhancement was desired.

The contrast and brightness of the image stack was adjusted to enhance the perception of the sampled endometriotic peritoneal lesion: this was generally made by changing the incline of the grey scale values for the dataset. Histogram equalisation (baseline subtraction in ImarisBasic 4.1visualisation

suite) (Bitplane, AG, Scientific Solutions Zurich, Switzerland) was also used to improve the contrast by a non-linear mapping of the grey levels in an image. This method frequently was utilised when the grey levels were concentrated in a small portion of the range of possible values.

It was important to realize that the application of filters to the dataset could ultimately affect the quantitative measurements of three-dimensional reconstructions that were produced from it. Therefore, the applications of filters in some cases were only used for qualitative purposes, and the quantitative measurements were made on the datasets that had been unprocessed.

Segmentation

Segmentation refers to the extraction procedure of a desired object (or objects) of interest (i.e. blood vessel and nerve fibre structures) from the background in an image or dataset. There are a variety of methods available that could be used to do this, such as those found in ImarisBasic 4.1 visualisation suite (Bitplane, AG, Scientific Solutions Zurich, Switzerland) (thresholding and masking).

The process of segmentation also was aided through manual intervention or automatically via software algorithms. Segmentation could either be performed prior the three-dimensional reconstruction by processing the images in the dataset, or post the three-dimensional rendering had been completed.

Addition of colour to the dataset

The photomultiplier tube was activated within the Leica Microsystems LAS AF-TCS SP5 integrated software (Leica Microsystems, Pty, Ltd, Wetzlar, Germany) and the colour for the fluorophore emission were selected for blood vessels (Red) and nerve fibres (green).

Three-dimensional reconstruction

After acquiring a Z-stack (or series), the data were processed using the maximum projection function in the Leica Microsystems LAS AF-TCS SP5 integrated software (Leica Microsystems Pty Ltd, Wetzlar, Germany) to produce a three-dimensional projection for quantitative interpretation.

Three-dimensional measurement

Filament Tracer

Third party volume rendering software ImarisBasic 4.1 visualisation suite and the module Filament Tracer (Bitplane, AG, Scientific Solutions Zurich, Switzerland) were utilised for quantitative measurements within the datasets. The filament-like structures were detected in two and three-dimensions which allowed for the manual drawing of the segments with automatic centering. Following a series of internal prompts, the image was processed to derive quantitative data.

Stereology

Stereology was used to provide a three-dimensional interpretation of two-dimensional cross sections of endometriotic peritoneal lesions collected as datasets using the laser confocal scanning microscope. The three-dimensional quantitative methods used were derived from modifications of published works [18-21].

Length density (Lv)

This was an estimate of the total length of all blood vessels and nerve fibres embedded in an accurately known volume of endometrial peritoneal lesion. This parameter is known as the length density (L_v) the total length of microvessels or nerve fibres per mm^3.

Branch point density (Bv)

This is known as the number of connections (branches) in the microvascular or neuronal network (per unit volume). A branching point (B_v) was defined as any vessel or neural segment within a section that had three or more distinct lumens or cut ends. Any capillary or nerve segment that appeared ambiguous for this criterion was not counted as a branching vessel or neuron **(Figure 1)**.

Segment length between branch points (L_V/B_V)

The segment length between branch points (L_v/B_v) was used as a relatively crude estimator obtained from two primary measurements, the length density (L_v) and branch point density (B_v). By dividing the sum of lengths of microvessels or nerve fibres/mm^3 (L_v) by the total number of connections (branch) points/mm^{-3} (B_v), the simplest average segment length of individual blood vessels or nerve fibres were obtained between successive branch points (L_v/B_v) **(Figure 1)**.

Capillary radial diffusion distance r(diff)

The capillary radial diffusion distance r(diff) is based upon the general recognition that capillaries are closely spaced in actively metabolizing tissue in order to minimize the effects of diffusion on the rate of supply of metabolites and on the rate of removal of waste products [29]. The r(diff) is an approximation that provides a simple, strong indication of a cylindrical zone of diffusion around a small vessel and this is estimated using L_v data [19,22]. Thus, the radius of this typical tissue cylinder ('diffusion radius'=r) including the radius of the capillary itself is then estimated as:

$$r\left(diff\right) = 1/\sqrt{\pi - Lc}$$

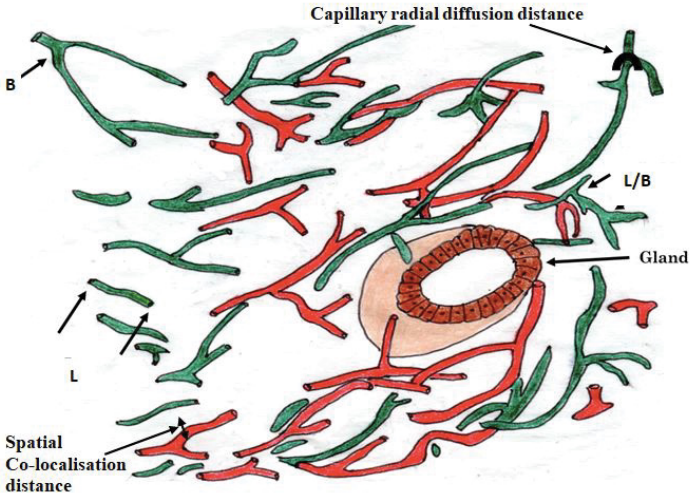

Figure 1: Illustrators rendition of the three-dimensional image reconstruction of a solitary gland and surrounding ($CD31^+$) blood vessels (Red) and ($PGP9.5^+$) nerve fibres (Green) in an endometriotic peritoneal lesion. The length densities (L), branch point density (B), segment length between branch point (L/B), capillary radial diffusion distance r(diff) and spatial co-localisation distance between blood vessels and nerve fibres are indicated.

Spatial co-localisation

Spatial co-localisation was the lineal distance measured between the blood vessel and nerve fibre.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) 24 (SPSS Inc. Chicago, Illinois, USA). Mean and standard error of the mean (SEM) are presented in **Figure 3**. The data were linearly related and normally distributed between blood vessels and nerve fibres were examined using the non-parametric Pearson's correlation coefficient (r). Results were considered to be statistically significant with p-values <0.05.

RESULTS

Quantitative analysis

Blood vessels

The total number of separate blood vessel structures observed was 608 with a length density (L_v) of each variable segment being 49.8 mm/mm^3 (range 35-67 mm/mm^3 ± 1.35 mm/mm^3 SEM). The total number of blood vessel structures observed to be branching were 1274, branch point density (B_v) 278 mm^{-3} (range 105-451 mm^{-3} ± 18.7 mm^{-3} SEM) with a segment length between branch points (L_v/B_v) of 179 µm (range 157-201µm ± 5.6 µm SEM). The capillary radial diffusion distance r(diff) for the blood vessels was observed to be 79µm (range 77-81 µm ± 1.5 µm SEM).

Nerve fibres

The total number of separate nerve fibre structures observed was 323 with a length (L_v) of each variable segment being 42.5 mm/mm^3 (range 26.5-53.3 mm/mm^3 ± 1.43 mm/mm^3 SEM). The total number of nerve fibre structures observed to be branching was 880, branch point density (B_v) 194 mm^{-3} (range 89-299 mm^{-3} ± 8.4 mm^{-3}) with asegment length between branch points (L_v/B_v) of 219 µm (range 193-245 µm ± 7.7 µm SEM).

Length density (Length per volume or L_V) of blood vessels and nerve fibres

The length density (L_v) data showed a consistent pattern throughout the endometriotic peritoneal lesions across the CD31$^+$ blood vessels and PGP9.5$^+$ nerve fibres. The density of small blood vessels (L_v) was 47% greater compared with the density of small nerve trunks and nerve fibre lengths. The blood vessel segments were 17.2% longer in comparison with the nerve fibre lengths **(Figure 2a)**. The longer vessel lengths (L_v) allowed for the potential for an increased number of branch point densities (B_v), to sustain the development of an adequate network of blood supply within the endometrial peritoneal lesion. There was no significant correlation between length densities for blood vessels and nerve fibres ($r=0.358$, $p=0.085$).

Branch point density (number per volume or B_V) of blood vessel and nerve fibre junctions or branches

The branch point density (B_v) of blood vessel and neural branch points were observed to have a 30.2% greater vessel density within the blood vessel group in comparison to the nerve fibres in the endometriotic peritoneal lesions **(Figure 2b)**. This increase is indicative of the formation of a complex blood vessel network which allows for a greater surface area of blood supply within the developing endometriotic peritoneal lesion. This is to ensure that every square micrometre of tissue is covered for the transportation of blood enabling the exchange of water and other molecules between blood and tissue. Facilitating the maintenance of the endometriotic peritoneal lesion, and the continual development of nerve fibres and an even greater coverage of blood vessels. There was a significantly strong positive correlation between blood vessel and nerve fibre junctions and branches ($r=0.876$; $p<0.001$).

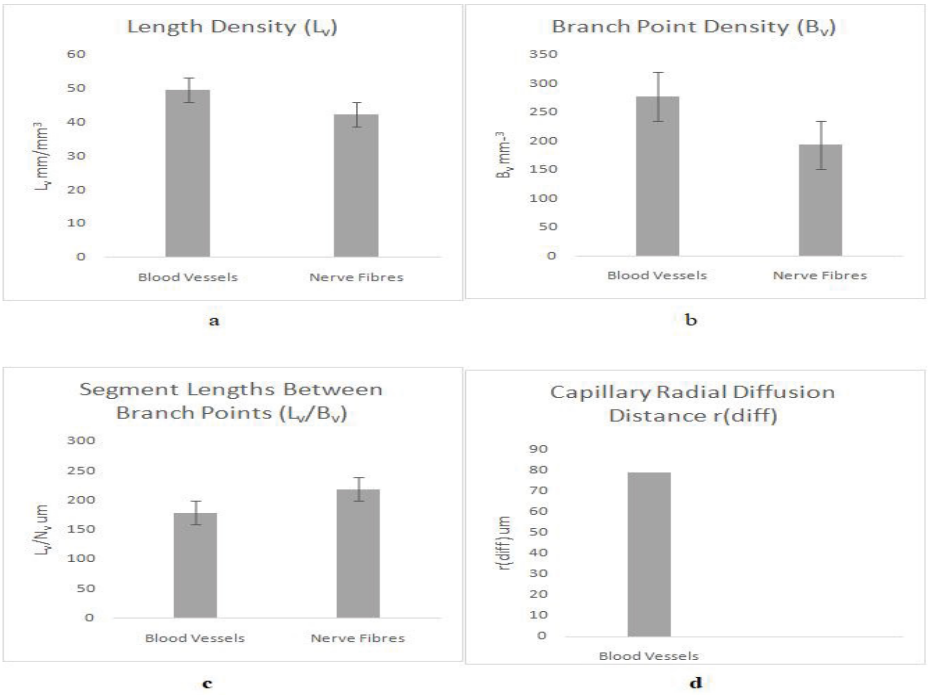

Figure 2: (a) Mean and standard error of mean (SEM) of length density (length per unit volume or L_v), **(b)** Branch point density (number per volume or B_v), **(c)** Segment length between branch points (L_v/B_v), **(d)** capillary radial diffusion distance r(diff) of endometriotic peritoneal lesions for (CD31$^+$) blood vessels and (PGP9.5$^+$) nerve fibres.

Segment length between branch point (L_V/B_V)

Segment length between branch points (L_V/B_v) for the blood vessels was a direct consequence of the longer length density (L_v) and a decrease in the numerical density of branch points (N_v). The average segment length data for blood vessels showed an 18.3% shorter distance compared with the nerve fibres **(Figure 2c)**. In a vascular or neuronal bed the segment length between branch points (L_V/B_v) is the estimation of the average distance between branch points of blood vessels and nerve fibres. Therefore, the average segment length between branch points (L_V/B_v), blood vessels and nerve fibres was determined by dividing the length density (L_v) of blood vessels and nerve fibres by the branch point density (B_v) [18-22].

Therefore, the short blood vessel segment lengths were a direct result of the longer length density (L_v) and an increase in the numbers of branch point density (B_v). Distances between each branch point provided an estimation of the spatial distribution as a function of distance along a blood vessel or nerve fibre. The shorter L_V/B_v were suggestive of the formation of a complex network of blood vessels. There was no significant correlation between blood vessels and nerve fibres (r=0.128; p=0.552).

Capillary radial diffusion distance r(diff)

The capillary radial diffusion distance r(diff) in the blood vessels in the endometriotic peritoneal lesion was observed to be 79μm (range 77-81 μm ± 1.5 μm SEM) **(Figure 2d)**. The capillary radial diffusion distance r(diff) measurement was indicative of the radius or 'cylinder' that the blood vessel occupied within the surrounding endometriotic peritoneal lesion tissue in order to facilitate the passive transport of oxygen, carbon dioxide, the osmosis of water and removal of waste products.

Spatial co-localisation distance between blood vessels and nerve fibres

The spatial co-localisation distance between ($CD31^+$) blood vessels and PGP9.5+) nerve fibres was found to be 15.4 μm (range 7.2- 42.2 μm ± 0.8 μm SEM). Additionally, in conjunction with capillary radial diffusion distance the nerve fibres fell within range for the facilitation of the physiological transfer of substances and transmission of electrical signals to coordinate tasks within the endometriotic peritoneal lesion. In addition, the spatial co-localisation distance supports that nerves are generally bundled alongside blood vessels, since the neurons of a nerve trunk have fairly high energy requirements.

Qualitative analysis

Illustrated in **Figure 3 (a-c)** are orthogonal maximum projections of blood vessels (red) and nerve fibres (green) in peritoneal endometriotic lesions, each occupying a total volume of 0.77 mm (x) x 0.77 mm (y) x 0.050 mm (z)=0.030 mm^3. There are limited numbers of branching blood vessels and nerve fibres

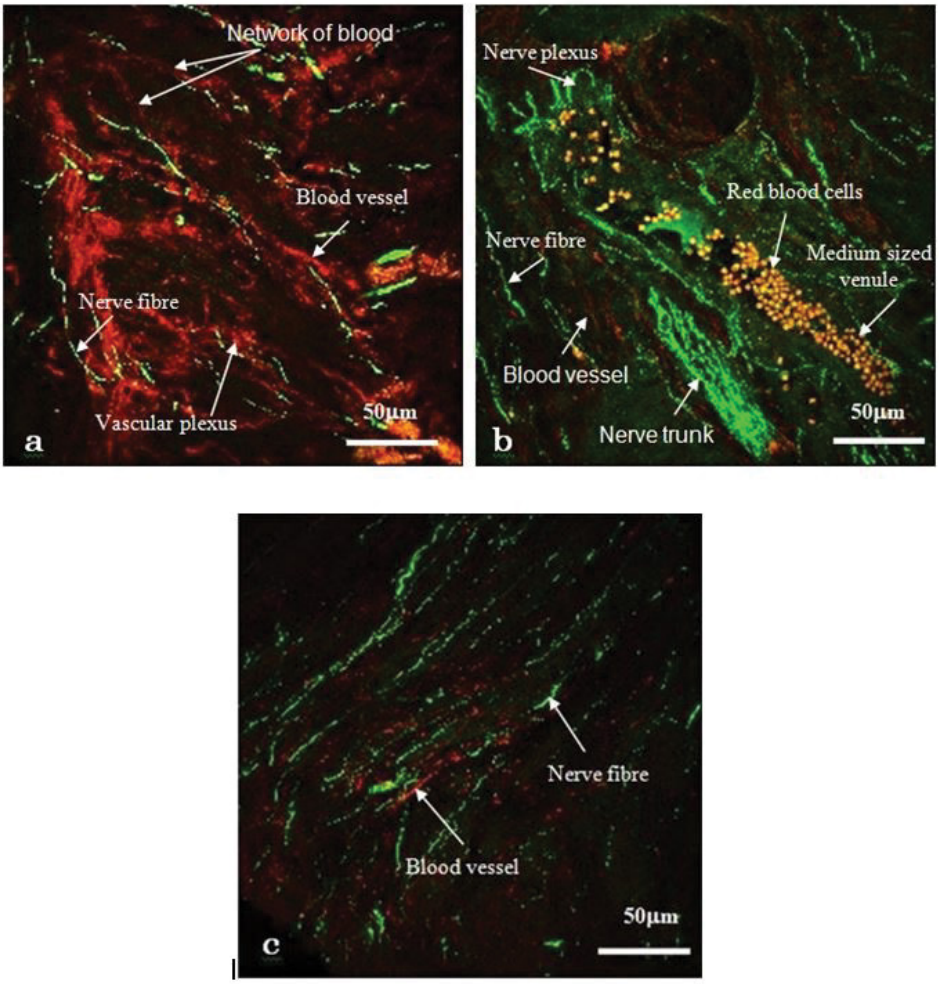

Figure 3: a-c, Orthogonal maximum projections of $CD31^+$ blood vessels (red) and $PGP9.5^+$ nerve fibres (green) in peritoneal endometriotic lesions, each occupying a total volume of 0.030 mm^3. (**a**) Network of capillaries and small veins drawing into vascular plexus and medium venule. Axons traversing alongside blood vessels. (**b**) Nerve trunk running alongside medium-sized venule. Yellow staining are Red Blood Cells within a medium sized venule. (**c**) Network of nerve fibres and blood vessels running parallel. Although the branching is limited it is regular and even in all directions between individual blood vessels and nerve fibres.

and their appearances are remarkably uniform in shape and diameter throughout the sections. Even though the branching is limited, it is regular and even in all directions between individual blood vessels and nerve fibres. The individual blood vessel segment lengths that make up the inter-branching portions are clearly distinguishable.

DISCUSSION

This feasibility study has demonstrated three-dimensional parallel relationships in peritoneal endometriotic lesions between small blood vessels and small nerve trunks and axons which are long slender projections of nerve cells, or neurons, that typically conduct electrical impulses away from the neuron's cell body. Blood vascular densities were 53.1% greater in comparison to the nerve fibre density. Vascular networks were visualised formed by frequent anastomoses between capillaries and small veins which drained into the vascular plexus and medium venules. In addition, the observation of branching networks of intersecting nerves forming a nerve plexus (resembling 'cables') which were composed both of afferent and efferent fibres (similar to individual 'wires') which arose from the merging blood vessels.

Length densities (L_v) in peritoneal endometriotic lesions were observed to be longer within blood vessels in comparison with nerve fibres. The branch point densities (B_v) were shorter within blood vessels compared to nerve fibres. The average segment length per branch point distances (L_v/B_v) were shorter for blood vessels. These were a direct consequence of the longer length densities (L_v) and a decrease in distance within the branch point density (B_v). This is a provision for a greater surface area of microvessels within the peritoneal endometriotic lesion. Aiding in the facilitation of the exchange of metabolites and waste products within the peritoneal endometriotic lesion. In contrast, a longer L_v/B_v such as with the nerve fibres would imply that is less complex and perhaps is still in the formative stages, reflecting less demand by the endometriotic peritoneal lesion for the supply of nutrients and metabolites at that point in time. The capillary radial diffusion distance r(diff) in conjunction with the co-localisation distance between blood vessels and nerve fibres demonstrated that the nerve fibres fell within the minimum range to facilitate the physiological transfer of substances and transmit electrical signals for the coordination of tasks within the endometriotic peritoneal lesion.

Our spatial co-localisation distance finding was supported by Asante and Taylor [30], the patterning and branching of vessels and nerves with signals from each influencing the migration of the other. The parallel growth of blood vessels and nerve fibres (neurogenesis) occurs in embryological development, this concept has been described in pathological processes such as cancer [13] and endometriosis [30].

There is mounting evidence indicating that ectopic endometriotic lesions recruit their own unique neural and vascular supplies through a process called neuroangiogenesis [30]. It is believed that these nascent nerve fibres in endometriotic lesions influence dorsal root neurons within the central nervous system, potentially influencing an increased pain perception in patients.

In a concurrent study Asally [31] observed that neurotrophins and neuronal guidance molecules and their receptors were synthesised *in-situ* within peritoneal ectopic lesions. Applying neurotrophic factors; Glial cell-derived neurotrophic factor (GDNF), Persephin, NT-3 and NT-4 were found to have been expressed in the endometriotic peritoneal lesions. These molecules were found to be highly expressed in the glands of the endometriotic peritoneal lesions, which may facilitate the growth and maintenance of nerve fibres and offering an explanation of the higher density of nerve fibres in the stroma near the endometriotic glands. In addition, the presence of ectopic lesions within the peritoneal cavity may affect the environment, and in turn, the peritoneum altered appeared to play a role in the growth of nerve fibres and their development and maintenance in peritoneal lesions. Within the endometriotic peritoneal lesions the neuronal guidance molecules, Semaphorin 3E and Slit-2 and their receptors Plexin D1 and Robo 4 were also found to be significantly highly expressed in the glands of endometriotic peritoneal lesions.

The role of guidance cues that Semaphorins play in nerve development are dual acting in attracting or repelling axons which are dependent upon the receptor bind and on the crosstalk between Semaphorin receptors and other pathways, whereas with blood vessels the receptor Plexin D1 and Semaphorin 3E interaction appears to mediate repulsive vessel guidance [32,33]. The growth of both blood vessels and nerve fibres are closely integrated processes, which are linked by common pathways and molecules [13,33,34].

The endometrium has intrinsic angiogenic potential and endometriotic lesions tend to grow in areas with rich vascularisation, this is suggestive that angiogenesis plays a crucial role in the establishment, development and growth of endometriotic lesions [35,36]. It also supports the ongoing lesion growth and progression, as demonstrated in several rodent study models of endometriosis [30]. There is a range of angiogenic proteins synthesized in endometriotic lesions [36].

Both *in-vivo* and *in-vitro* studies have identified VEGF-A as a crucial molecule secreted from the cutaneous nerves that trigger arterial differentiation of nerve-associated vessels, however, nerve-derived VEGF is dispensable for the initial recruitment of blood vessels along the nerve [37,38]. The most potent and specific angiogenic factor VEGF-A is strongly expressed in endometriotic lesions [39-45]. It has effects on endothelial cell proliferation, migration, organisation in tubules and enhanced permeability, all of which participate in the angiogenic cascade [14,46]. The maintenance of angiogenesis in endometriotic lesions is achieved with high concentrations of VEGF-A in the peritoneal fluid [47]. VEGF-A expression in endometriotic lesions has been described as higher than in eutopic endometrium [39,40,42].

Activated peritoneal macrophages and neutrophils also have the capacity to produce and secrete VEGF. The density of macrophages in endometriotic peritoneal lesions was greater compared to control peritoneum from women without endometriosis [48]. Neuropilins, which were initially described as semaphorin receptors responsible for neuronal guidance, also have nanomolar affinities for VEGF isoforms which can mediate angiogenesis [49]. Other factors including secretoneurin, a neuropeptide expressed in nerve fibres has been found in close apposition to blood vessels that stimulates endometrial cell migration and angiogenesis in in-vitro and in-vivo assays [50].

Blood vessels also promote cues for the growth and alignment of adjacent nerves. The patterning of the nervous system is achieved through co-ordinated actions of a variety of repulsive or attractive neuronal guidance factors that direct the growth of growing axons to specific pathways [51]. Asante and Taylor [30] proposed the theory of neuroangiogenes is within endometriotic peritoneal lesions to explain the common coincidence of nerves and blood vessels traveling together into new tissues. Nerves and blood vessels do sometimes travel together as they traverse subepithelial tissues, this is evident in the computer reconstructed datasets collected via laser scanning confocal microscopy in our study. However, Hey-Cunningham et al. [36] have reported a divergence as they enter the lesion, this type of divergence has been observed as nerves enter eutopic endometrium in association with a highly significant decrease in the expression of neuropilins (axonal and vascular guidance molecules and receptors) for VEGF in eutopic endometrium.

Miller and Fraser [17] have hypothesised that these nerve fibres in endometriotic lesions develop as neurites, which branch out from pre-existing neurons, and are stimulated by nerve growth factor acting on the high-affinity Trk-A receptors. Kandel [52] reported that specific molecular cues guide axons to their targets. Where each newly-growing nerve fibre has an amoeboid tip that projects searching pseudopodia into the interstices of the tissue through which the fibre is growing. Each tip moves along, guiding its fibre behind it.

Relatively little is known about the pathophysiological mechanisms of endometriosis and so far, endometriosis continues to remain a significantly under diagnosed and under-treated disease [53]. A better understanding of local neurogenesis and angiogenesis, and the role nerves play in pain generation is required and is a critical element to the development of improved treatment [17].

Yan [54] reported that the neural densities in endometriotic lesions were increased. These endometriotic lesions secrete neurotrophic factors (NTFs) that promote intralesional neural sprouting and hypertrophy and induce neural remodelling additionally, hyperinnervation in eutopic endometrium has also been demonstrated however, this hyperinnervation appearance is not exclusively been observed to be present in endometriosis [54]. Endometriotic lesions are primarily wounds undergoing repeated tissue injury and repair [55]. Yan [54] postulated that NTFs, neurotransmitters and neuropeptides were secreted by intralesional nerve fibres in turn these would affect the behaviour and functions of endometriotic lesions. Further adding that nerve fibres and endometriotic lesions were truly partners in crime regarding pain experienced by women with endometriosis.

CONCLUSION

This study has demonstrated the possibilities of three-dimensional visualisation utilising immune histochemistry, laser scanning confocal microscopy and computer reconstruction of blood vessels and nerve fibres in peritoneal endometriotic lesions. Inclusions for future studies are localisations studies performed with more specific markers for sensory C, sympathetic and parasympathetic neurons,

AUTHORS' NOTE

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DECLARATION OF CONFLICTING INTERESTS

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