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A double blind study of the neuropathology in goats exposed to hyperbaric conditions

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A double blind study of the neuropathology in goats exposed to hyperbaric conditions

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HSE BOOKS

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Introduction.

Goats have been used as an animal model to study the effects of decompression following hyperbaric exposure since the experiments performed by J.S.Haldane and others, from which the first decompression tables were derived at the beginning of the last century (Edmonds, *et al*., 1981). Their suitability for such studies lay in their similar physical size to human subjects, their ease of handling and husbandry, similarity of lesion pathology and the establishment of a range of easily recognisable clinical signs which appear to correspond well with recognised signs of the condition in man (Palmer,1997). These signs have been used to create an arbitrary classification of syndromes exhibited by goats into types I and II decompression sickness in a similar way to the classification used in man (see Table 1).

A number of studies have been carried out on material made available from goats subjected to decompression procedures (Palmer,1990) and euthanased at various time points after decompression in order to unravel the sequence of events and mechanisms in the pathogenesis of observed lesions associated with spinal decompression sickness. These studies, carried out from 1972 (Palmer, *et al.,* 1976; 1978) demonstrated similarities to lesions observed in spinal cord from exposed humans and elucidated the nature, distribution and evolution of these lesions providing an important reference source for this study. Comparisons made with conditions in other species, both natural and experimental (Palmer,1986) provide compelling evidence that the primary spinal cord lesions are infarcts in the white matter.

These previous studies failed to detect significant lesions in the brains of affected goats despite the presence of vascular brain pathology in affected humans (Palmer, *et al.,*1992). It was suggested that the significant differences in the blood supply to the brain between the two species may account for this difference.

Table 1; Clinical signs of decompression sickness in goats and man.

Subject	Type I (minor)	Type II (serious or spinal)
Goat	Scratching, skin nibbling, lifting legs, stamping feet, lameness.	Dyspnoea, limb paresis, quadriplegia.
Man	Pruritus, skin rash, joint pain (bends), localised swelling.	CNS disorder (paresis or paraplegia), respiratory symptoms (chokes), other manifestations.

In the series of studies carried out on goats since 1972 observations of neuropathology were made with knowledge of the exposure history for each goat. In the previous studies this knowledge was important to assist in the interpretation of examined material, however, this knowledge has the potential for observer bias in the interpretation of the significance of mild pathology. This took the form of an increase in the incidence of diffuse axon damage. Recently, concerns have been raised that repeated decompression, even in the absence of any clinical signs of decompression sickness, may be associated with some degree of central nervous system (CNS)

pathology. A number of goats exposed to numerous simulated dives by the Defence Evaluation and Research Agency (DERA) had reached the end of their natural lives and were planned to be culled. The Health and Safety Executive (HSE) took this opportunity to organise the examination of material obtained from these animals. Some of these goats had experienced, and had been treated for, decompression sickness while others had shown no clinical signs. They therefore formed a cohort of animals in which it might be possible to determine if repeated decompression was associated with an accumulation of CNS pathology. In order to remove observer bias the study was designed in such a way that the material was to be examined without knowledge of the decompression history of the goats. This blinded approach was considered important because the pathology present could be subtle and thus had the potential to be confused with changes that may be present in normal animals. In addition, to increase the sensitivity of detection of changes it was decided to use glial fibrillary acid protein (GFAP) immunohistochemistry since this provides a sensitive method for demonstrating insults to the CNS.

Methods.

Goats

Formaldehyde-fixed post-mortem material was supplied to Cambridge from a total of 36 goats. The tissue included the entire brain and spinal cord plus a selection of samples from other tissues together with clinical pathology data and details of gross pathology.

On arrival the samples were allocated code numbers distinct from their original identification used by the sampling laboratory. The proportion of exposed to control animals was undisclosed and remained so until after the CNS tissue had been examined. Table 2 summarises details of age and exposure history of decompression exposure supplied after tissue had been evaluated for pathological changes.

Tissue sampling

The spinal cord for each goat was sectioned transversely at the level of each spinal nerve root entry zone. A block of tissue approximately 3 mm thick was then taken at the level of each spinal nerve root to provide a series of transverse blocks. The remaining tissue was sectioned longitudinally along the midline to provide two longitudinal blocks which were embedded so that with one block the medial surface was sectioned while with the other the lateral surface was sectioned (Fig.1). A total of 81 sections of spinal cord were processed which resulted in a total of 3132 sections being examined.

Seven blocks were prepared from the brain of each goat at the following locations:-

1.) A rostral transverse cerebral block to include the olfactory tract, the head of the caudate nucleus and the genu of the corpus callosum.
2.) A middle transverse cerebral block just caudal to the optic chiasm, including the hypothalamus and amygdala.
3.) A caudal transverse cerebral block at the level of the pineal gland to include the hippocampus.
4.) A transverse block through the mid-brain at the level of the rostral colliculi.
5.) A median saggital block prepared from the right half of the cerebellum removed from the underlying pons and medulla.

6.) A transverse block through the remaining cerebellum and underlying medulla.
7.) A transverse block at the level of the obex.

All tissue blocks were paraffin embedded and 8 μm thick sections were prepared for staining with haematoxylin and eosin (H&E) or for GFAP immunohistochemistry.

Immunohistochemistry.

Paraffin sections from transverse blocks at seven levels of the spinal cord (third and sixth cervical, second and seventh thoracic and third, fourth and fifth lumbar) were prepared on Vectabond coated slides for immunohistochemistry following standard procedures and air dried for a minimum of 48 hours at 37ºC. Sections were cleared in xylene for 5-7 minutes, rehydrated through alcohols and washed twice in distilled water. Endogenous peroxidase activity was blocked by incubation in 0.6% hydrogen peroxide in methanol for 10 minutes. Antigen retrieval was performed by treating sections with 0.1% trypsin in phosphate buffered saline (PBS) for 10 minutes at room temperature. Non-specific binding activity was blocked 10 % casein in PBS for 5 minutes. Polyclonal rabbit anti-cow GFAP (Dako) was applied as primary antibody at a 1:500 dilution overnight at 4ºC. Sections were washed in PBS with 0.1% Triton added three times for 5 minutes each. Incubation with biotinylated goat-anti-rabbit secondary antibody was performed for 45 minutes at room temperature before washing again as above An avidin-biotin-peroxidase detection system was used (Vector ABC reagent kit using 2 drops of solution A and 2 drops of solution B in5 ml PBS and applied to slides for 30 minutes at room temperature) and peroxidase activity was demonstrated using a diaminobenzidine (DAB) substrate detection kit (Vector). Slides were then dehydrated through alcohols, cleared in xylene and mounted with DPX.

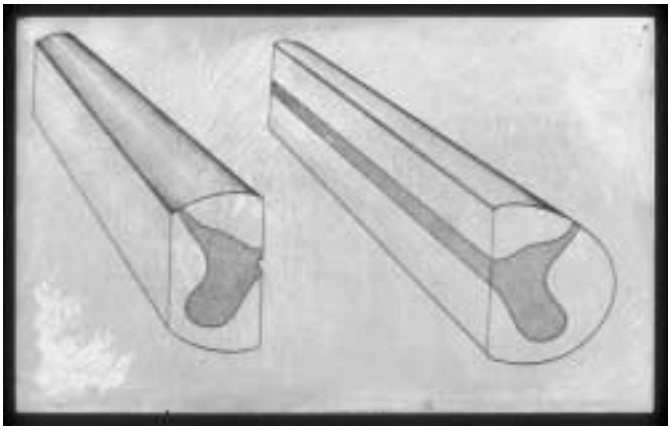

Figure 1: Spinal cords were sectioned at the level of each spinal nerve root entry zone producing transverse blocks. Intervening spinal cord was sectioned longitudinally producing two blocks as shown above which were embedded left hand side down to produce longitudinal sections of lateral, dorsal and ventral funiculi

Table 2; Summary of information supplied relating to diving history. Bold type in this table and tables 3 and 4 highlights animals with lesions consistent with previous episodes of spinal cord infarction.

DERA Code	Lab Code	DOB	First Dive	Last Dive	Type 1	Type 2	Dives
88 B	2/234	11/4/88	29/10/93	8/4/97	0	0	16
91 M	3/270	5/4/91	15/11/93	16/12/97	0	0	18
88 A	2/233	11/4/88	29/10/93	8/4/97	0	0	17
89 E	2/036	13/3/89	19/10/93	1/5/97	0	0	16
89 H	2/037	25/3/89	26/10/93	1/5/97	0	0	15
F 21	3/033	3/3/93	21/10/98	21/10/98	0	0	1
K 13	3/034	21/5/97	20/8/98	23/11/98	0	0	3
91 G	2/458	25/3/91	12/10/93	18/3/97	0	0	15
F 3	2/497	3/3/93	18/8/94	5/11/96	0	0	11
F 25	2/602	3/3/93	11/8/98	29/9/98	0	0	2
E 86	3/088	3/3/93	4/3/96	2/4/97	0	0	5
91 H	4/496	25/3/91	20/7/93	12/5/94	0	0	3
K 22	3/236	18/5/97	22/10/98	27/11/98	0	0	2
F 4	**2/496**	**3/3/93**	**18/8/94**	**23/11/98**	**23/11/98**	**0**	**17**
91 U	4/495	14/4/91	9/8/93	29/1/98	13/9/95-3	0	15
91 P	2/646	6/4/91	14/10/93	20/10/98	10/9/98-2	0	21
91 F	2/457	25/3/91	12/10/93	10/9/98	8/11/95-2	0	21
91 T	2/603	12/4/91	16/10/93	28/1/98	16/10/93?	0	20
E 94	**3/089**	**3/3/93**	**13/1/94**	**18/3/97**	**6/12/96**	**17/8/94**	**13**
K 23	3/237	18/5/97	22/10/98	22/10/98	0	22/10/98	1
89 C	2/647	13/3/89	9/9/93	10/9/98	4/11/94-2	0	19
F 11	**3/271**	**31/9/92**	**15/2/96**	**27/10/98**	**4/11/97**	**26/3/97**	**11**
91 N	3/428	6/4/91	14/10/93	28/1/98	28/1/98-4	0	12
90 A	3/429	8/4/90	26/10/93	19/3/97	29/11/93	0	15
91 K	3/487	5/4/91	7/10/93	9/9/98	9/9/98-2	0	21
91 V	3/488	14/4/91	19/8/93	3/12/97	13/12/93-2	0	8
91 B	3/610	23/3/91	15/2/93	2/12/97	15/2/93	0	10
E 93	2/334	21/8/90	7/11/94	3/9/97	27/4/97	3/9/97	13
88 C	2/335	13/4/88	12/8/93	1/12/97	12/8/93	27/6/94	15
90 E	3/708	5/5/90	19/10/93	20/3/97	19/10/93	0	15

Results

Procedure for evaluation of pathological changes

The most frequently observed pathological changes in H&E stained sections of the spinal cord were evidence of Wallerian degeneration in white matter and perivascular cuffing. The severity and frequency of these changes varied between sections and between animals and therefore the severity of these pathological changes was assigned a score. In H&E stained sections the following changes characterise Wallerian degeneration. In longitudinal section the characteristic changes are chains of vacuoles, a small number of which may contain "myelophages" while in transverse sections the change appears as single vacuoles containing "myelophages".

The extent of these changes was scored according to the following scale:-

0. No evidence of Wallerian degeneration.
1. Possible evidence of a small numbers of axons undergoing Wallerian degeneration.
2. Definite evidence of a small number of nerve fibre undergoing Wallerian degeneration.
3. Moderate frequency of scattered Wallerian degeneration in one or more funiculi.
4. Very frequent evidence of Wallerian degeneration in more than one funiculus.
5. Focal area of axon loss or prominent focal area of Wallerian degeneration.

Figs. 2 and 3 illustrate examples of different score levels. Each section was scored and a score recorded for each goat that comprised the sum of the scores of each of the longitudinal, or transverse, sections of spinal cord examined. The total score for all longitudinal and transverse sections for each goat is recorded in Table 3.

Astrocytes are very reactive cells and respond to changes in their immediate environment, therefore changes in the distribution and intensity of GFAP staining provide an indication that the CNS has been subjected to some insult.

Results of immunohistochemistry for GFAP on spinal cord sections were scored using a five point scale according to the following criteria:-

0. Negligible cell body labelling in white matter funiculi and a characteristic periaxonal, perivascular and subpial distribution of positively labelled cell processes (see Fig. 4). This was considered normal as it was the pattern observed in known normal animals.
1. Some cell body labelling apparent in one funiculus.
2. Cell body staining in more than one funiculus.
3. As 2 above and including evidence of changes in the distribution of labelled cell processes particularly around blood vessels in one funiculus.
4. Cell body labelling and distribution changes as described in 3 above but present in two funiculi.
5. Frequent cell body labelling and distribution changes in more than 2 funiculi.

Figure 4 shows examples of sections achieving different score levels. Astrocytosis scores for each goat comprised the sum of scores from each of the seven sections examined for each goat. In addition focal densities in the staining pattern were noted where observed.

Perivascular cuffing (see Fig.7) was also observed and its extent in different regions of the CNS (cerebrum, cerebellum, midbrain, medulla, cervical, thoracic and lumbar spinal cord) was scored using a five point scale reflecting frequency and thickness of the cuffs using the following scale:-

0 – None

1. Occasional subtle/equivocal increase in mononuclear cells in perivascular space.
2. Occasional vessel with unequivocal mononuclear infiltration surrounding blood vessels.
3. Moderate number of perivascular cuffs.
4. Frequent perivascular cuffs in focal regions.
5. Frequent diffuse prominent perivascular cuffs.

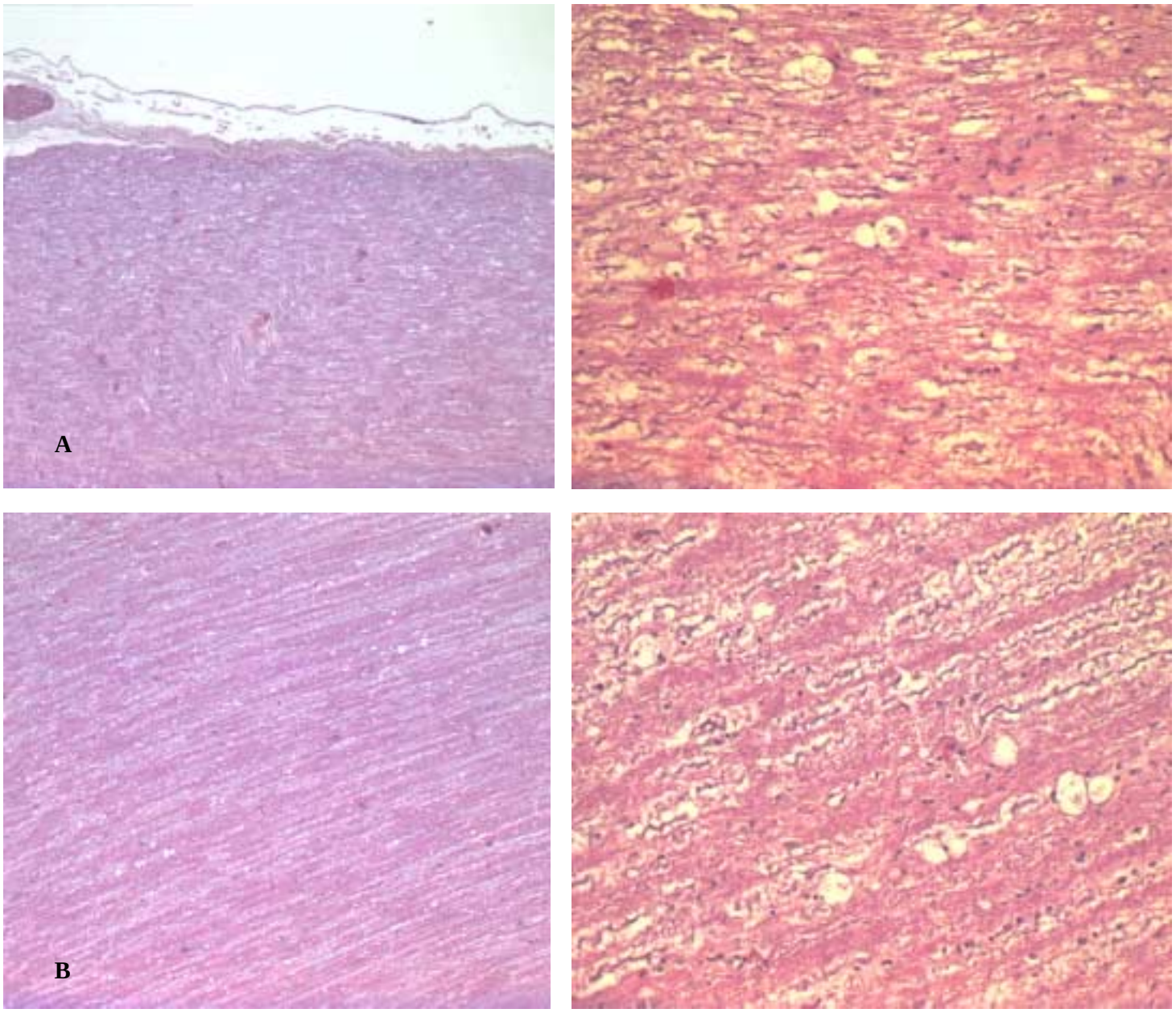

Figure 2A and 2B: Longitudinal H&E stained sections of spinal cord showing typical levels of Wallerian degeneration scored in the study. Low and higher power view of a field scoring:- A = 0; B = 3.

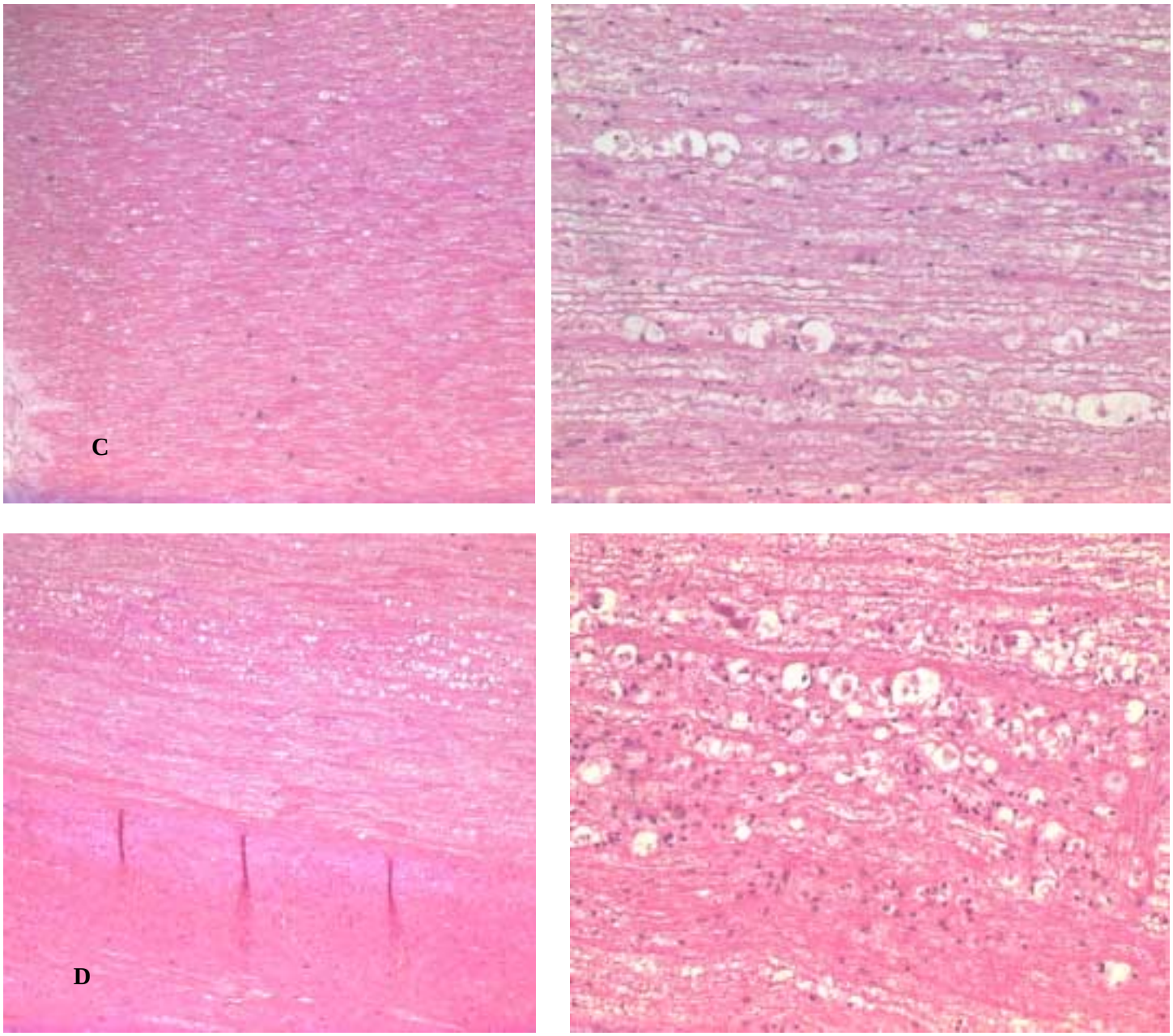

Figure 2C and 2D: Longitudinal H&E stained sections of spinal cord showing typical levels of Wallerian degeneration scored in the study. Low and higher power view of a field scoring:- C = 4; D = 5.

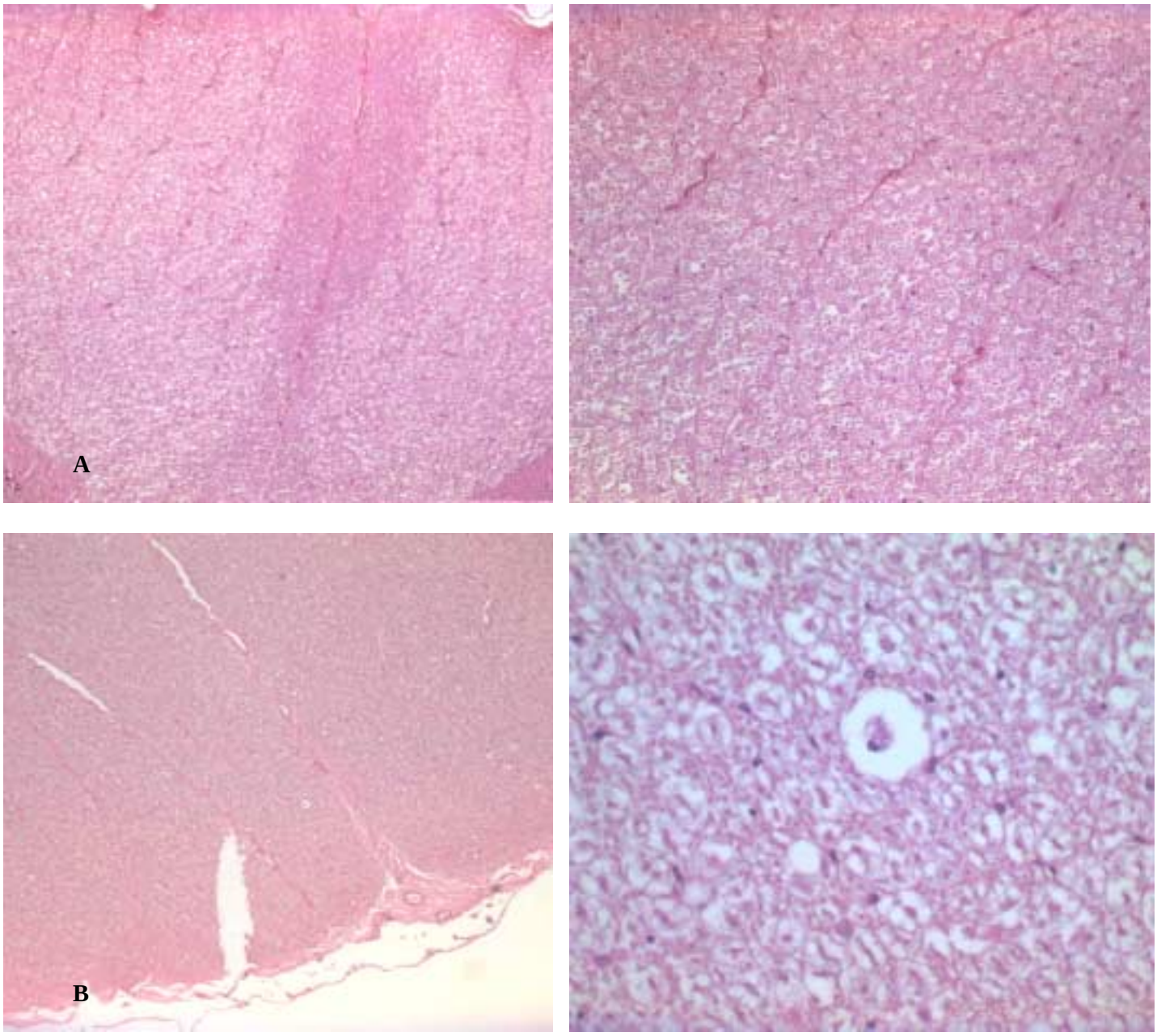

Figure 3A and 3B: Transverse H&E stained sections of spinal cord showing typical levels of Wallerian degeneration scored in the study. Low and higher power view of a field scoring:- A = 0; B = 3.

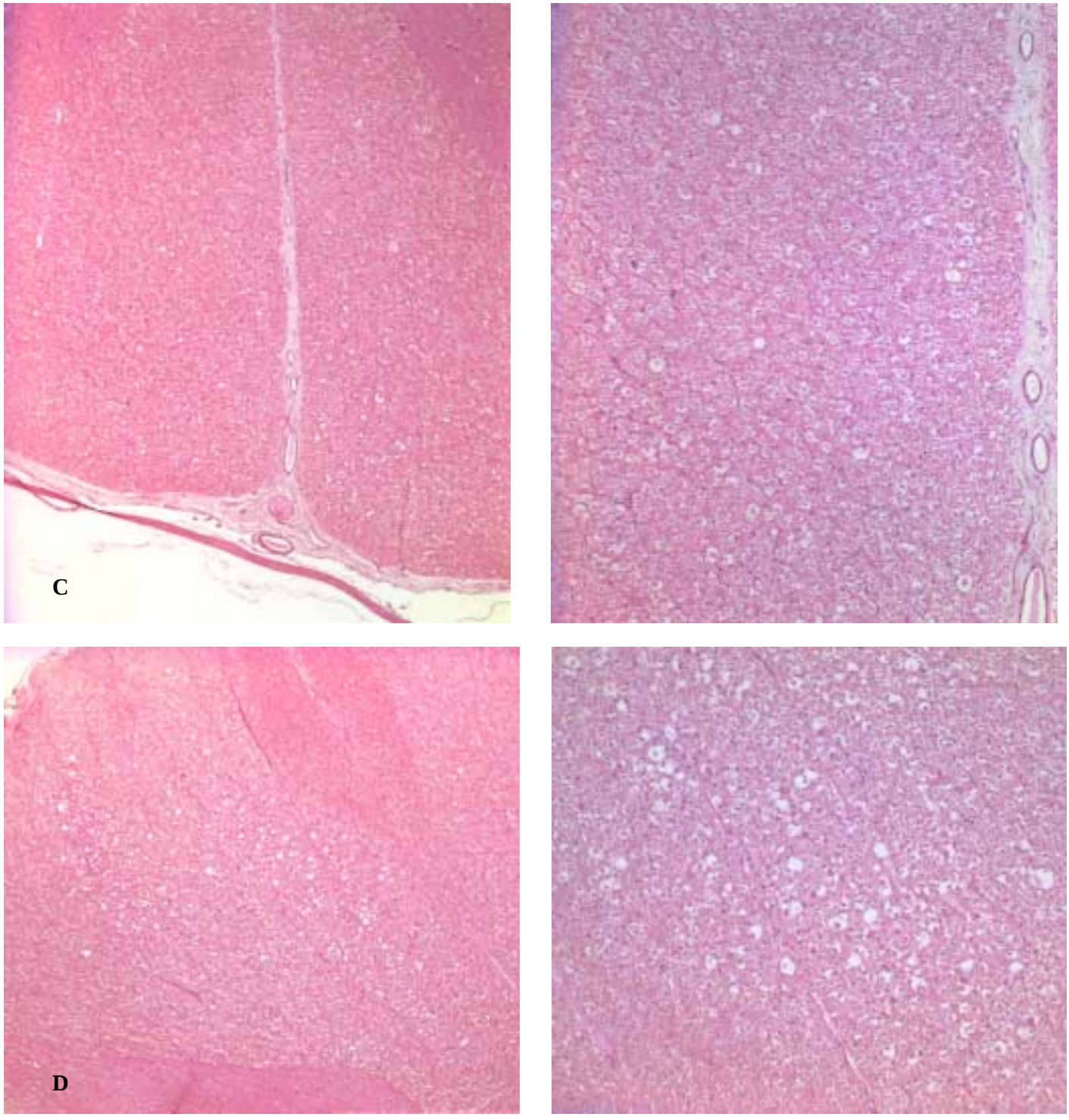

Figure 3C and 3D: Transverse H&E stained sections of spinal cord showing typical levels of Wallerian degeneration scored in the study. Low and higher power view of a field scoring:- C = 4 and D = 5.

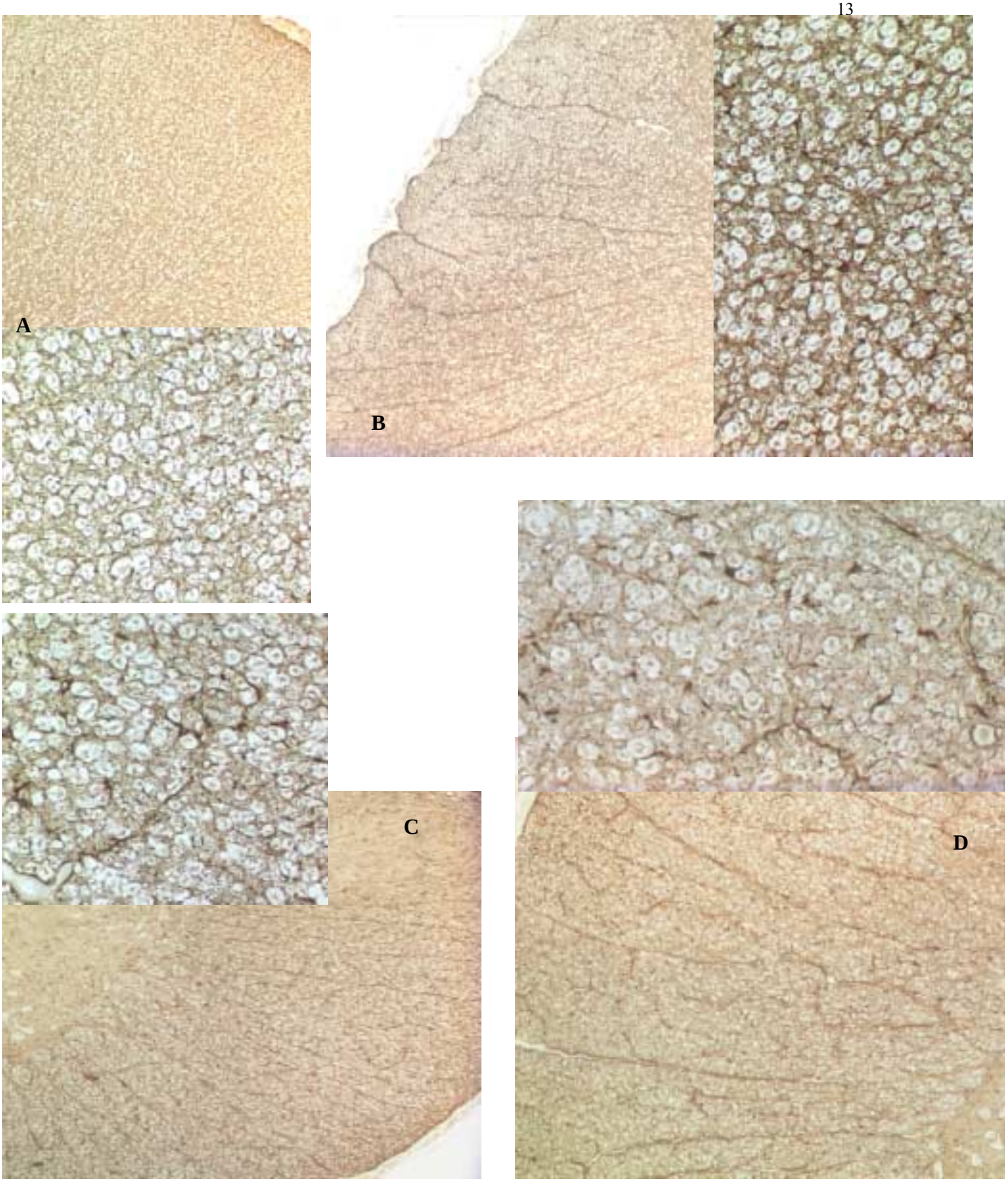

Figure 4: Low and higher power views areas of transverse sections of white matter labelled for GFAP by immunohistochemistry. Typical fields scoring 0 (A), 2 (B), 4 (C), 5 (D) are shown.

Table 3; Results of scoring for Wallerian degeneration in both longitudinal and transverse sections, astrocytosis and perivascular cuffing totalled across all sections examined for each goat as described in the text.

DERA Code	CPL Code	W. D. (LS)	W.D. (TS)	Astrocytosis	Periv. Cuff.
88 B	2/234	84	3	0	4
91 M	3/270	91	29	21	4
88 A	2/233	125	37	3	12
89 E	2/036	57	0	1	10
89 H	2/037	28	0	0	4
F 21	3/033	34	12	19	0
K 13	3/034	14	0	16	2
91 G	2/458	70	18	12	2
F 3	2/497	56	21	23	1
F 25	2/602	38	0	25	6
E 86	3/088	39	18	9	1
91 H	4/496	101	27	22	3
K 22	3/236	54	3	30	6
F 4	**2/496**	**97**	**35**	**14**	**3**
91 U	4/495	112	15	17	3
91 P	2/646	59	12	15	1
91 F	2/457	56	9	15	8
91 T	2/603	39	3	17	4
E94	**3/089**	**67**	**19**	**23**	**2**
K 23	3/237	31	0	27	0
89 C	2/647	62	12	20	9
F 11	**3/271**	**84**	**14**	**23**	**10**
91 N	3/428	135	33	12	4
90 A	3/429	116	39	13	4
91 K	3/487	77	6	14	17
91 V	3/488	101	39	19	8
91 B	3/610	92	36	13	8
E 93	2/334	43	6	16	3
88C	2/335	123	30	16	3
90 E	3/708	114	30	9	16
F 33	4/451	83	9	26	4
F 30	4/452	69	6	24	2
F 32	3/611	113	45	16	2
F 35	3/707	73	6	4	6
F 27	4/529	41	3	26	0
F 26	4/530	32	6	8	1

A. Dived, no decompression illness. **Control.**

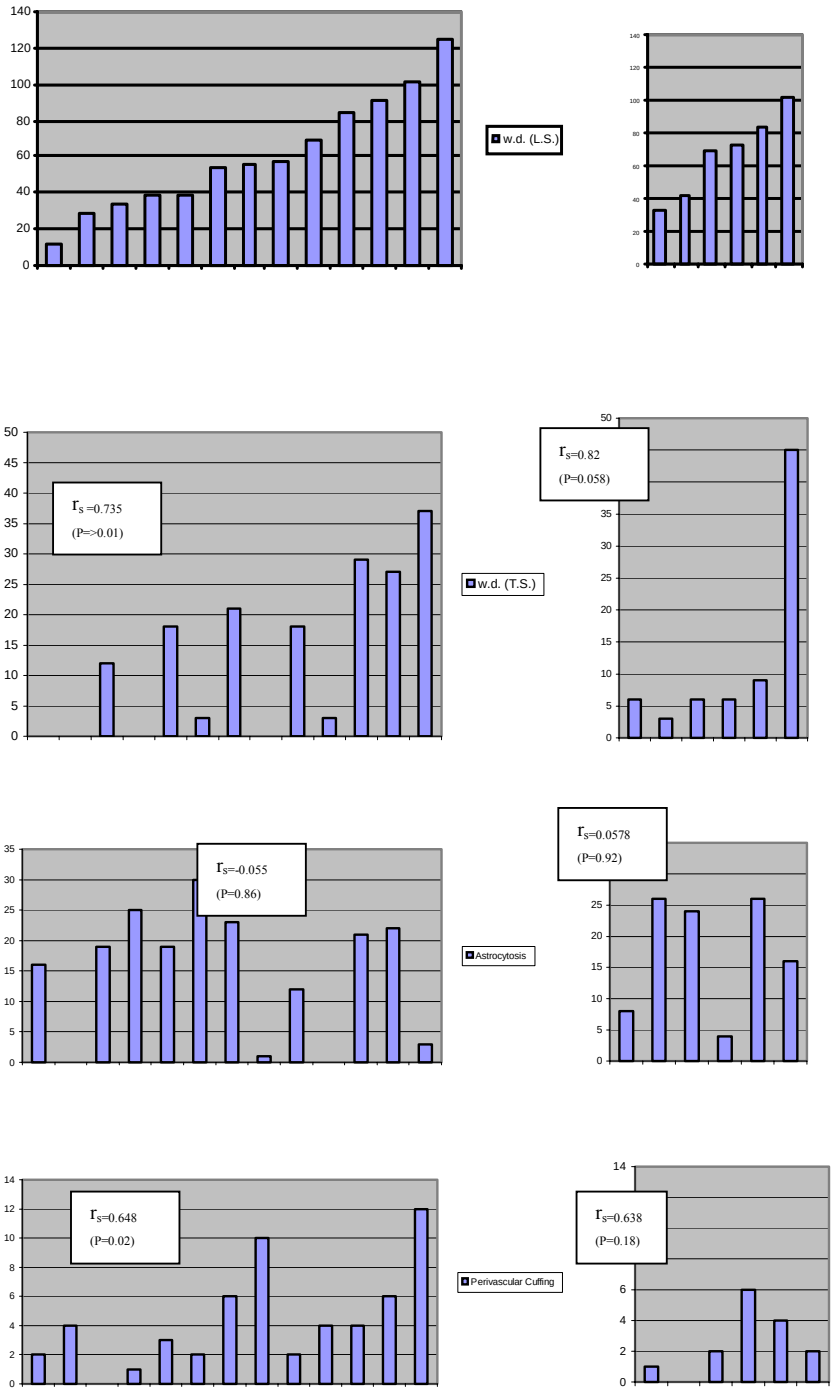

Figure 5 A. Bar charts showing scores for goats for each of four scored pathological criteria for exposed goats without clinical signs compared to control goats.
Each bar represents an individual goat. Goats are ordered with respect to increasing levels of Wallerian degeneration detected on longitudinal sections of spinal cord.
Correlation of other parameters with Wallerian degeneration (in longitudinal section) is indicated by Spearman Rank Correlation Coefficients stated the first time those results appear.

B. Dived, decompression illness. **Control.**

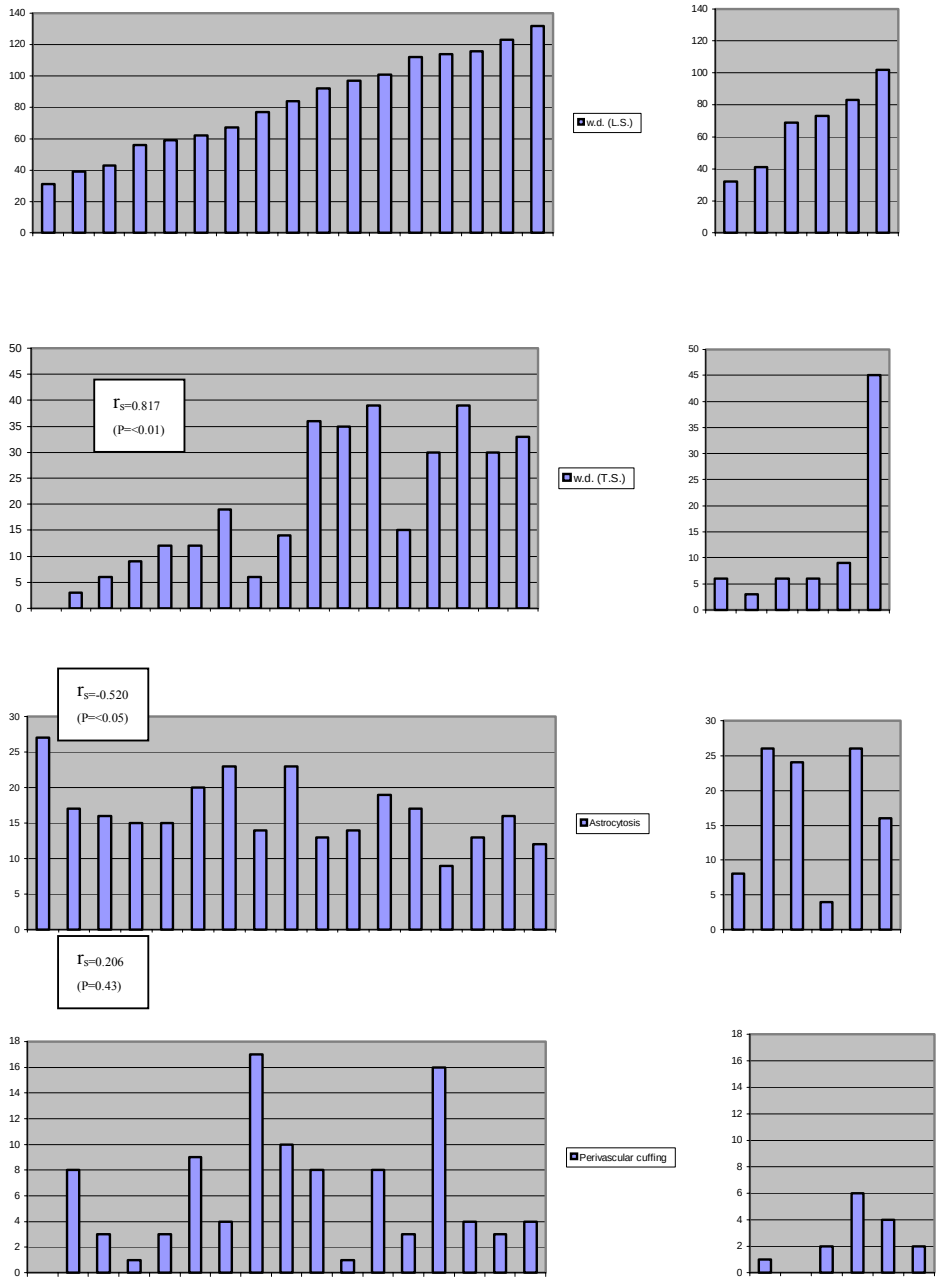

Figure 5 B. Bar charts showing scores for goats for each of four scored pathological criteria for exposed goats with clinical signs compared to control goats.
Each bar represents an individual goat. Goats are ordered with respect to increasing levels of Wallerian degeneration detected on longitudinal sections of spinal cord.
Correlation of other parameters with Wallerian degeneration (in longitudinal section) is indicated by Spearman Rank Correlation Coefficients stated the first time those results appear.

C. Dived, decompression illness. **Dived, no decompression illness.**

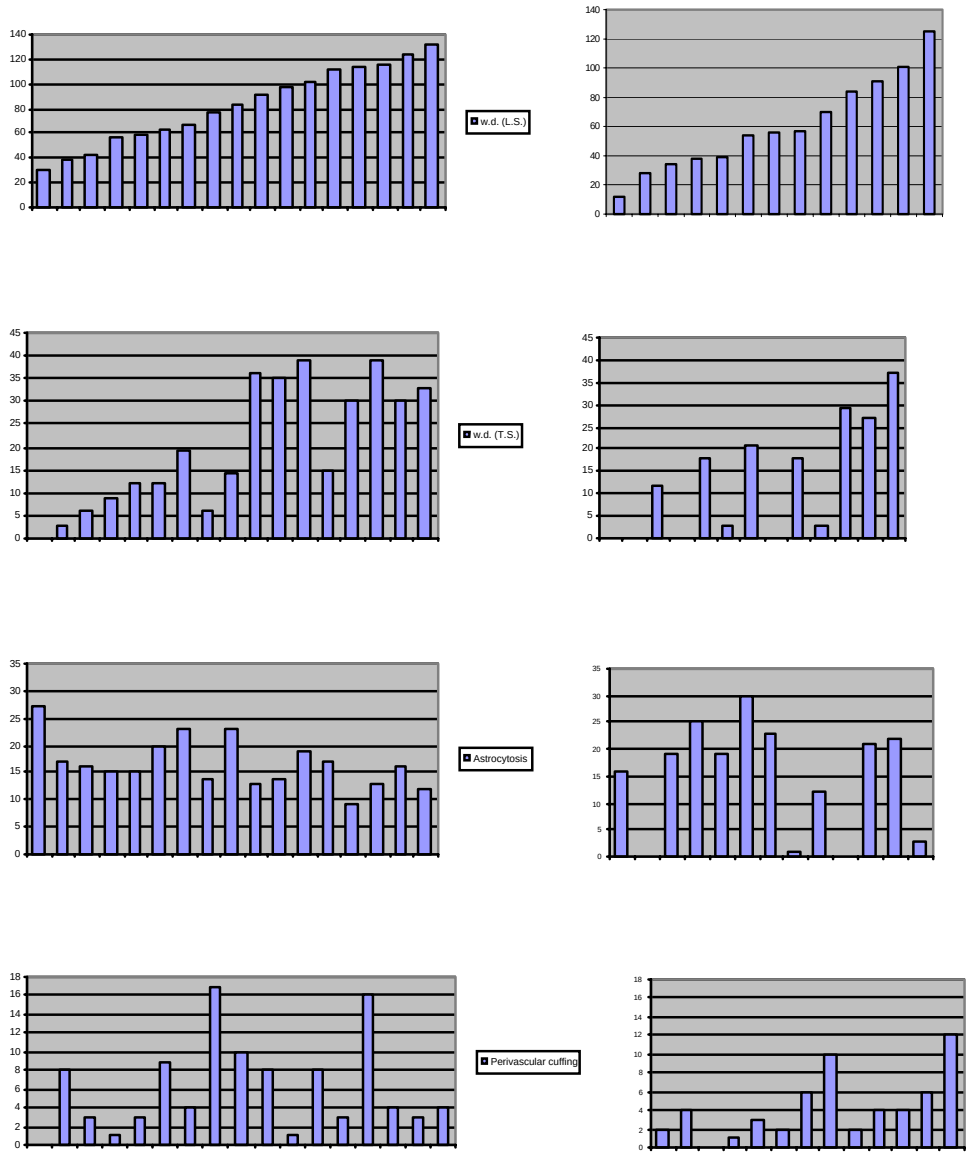

Figure 5 C. Bar charts showing scores for goats for each of four scored pathological criteria for exposed goats with and without clinical signs compared to control goats.
Each bar represents an individual goat. Goats are ordered with respect to increasing levels of Wallerian degeneration detected on longitudinal sections of spinal cord.
There is no significant difference between the groups for any parameter scored.

Analysis of data obtained by analysis of spinal cord sections.
Before receiving the information on the decompression history of the goats the Wallerian degeneration and GFAP scores were analysed to determine if there was any relationship between the values obtained for individual animals. No correlation was found (r_s = -0.196; P = 0.252), similarly there was no trend for any grouping of the data that might indicate that there were 3 populations, i.e one with low scores (normal), one with medium scores (exposed but no symptoms) and one with high scores (exposed with symptoms).

The history of the goats was therefore requested and in Table 2 and Fig. 2 the pathological scores for each group of goats is recorded and plotted. It can be seen that there is a large amount of variation in scores within each group and for each parameter investigated. However, the highest scores for astrocytosis, Wallerian degeneration (in longitudinal spinal cord sections) and perivascular cuffing were attained by goats exhibiting signs of decompression illness. Apart from perivascular cuffing, the lowest scores did not always fall within the control group. Indeed, one control animal (3/611) showed high Wallerian degeneration scores and three others scored highly for astrocytosis. Additionally some exposed animals showed low scores for all measured parameters. Spearman Rank correlation coefficients were determined comparing Wallerian degeneration scores in longitudinal sections with other scored parameters. Correlation coefficients are shown on the relevant graphs in Fig. 2A and B. Significant correlations ($P<0.05$) were found between the scores for Wallerian degeneration in longitudinal and transverse sections (except for the control group) and between scores for Wallerian degeneration in longitudinal sections and perivascular cuffing in the exposed group not showing decompression illness symptoms.

Non-parametric statistical analysis showed no significant difference between any of the groups for any of the parameters measured (Kruskal-Wallis test) - see Table 4.

Table 4: Comparison of treatment groups of goats using the Kruskal-Wallis test. (Results were adjusted for ties. $P \leq 0.05$ required to demonstrate a significant difference between any of the groups.)

	H values	P value (2 degrees of freedom)
Wallerian degeneration (L.S.)	3.68	0.16
Wallerian degeneration (T.S.)	3.12	0.21
Astrocytosis	0.44	0.80
Perivascular cuffing	3.01	0.22

Table 5: Table relating age, sex and dive history parameters to Wallerian degeneration and astrocytosis parameters measured in the study.

DERA Code	Lab Code	W. D. (LS)	Dives	Last Dive	Post mortem date	Interval from last dive (months)	Astro - cytosis	Age (Yrs)	Sex
88 B	2/234	84	16	8/4/97	9/2/99	22	0	11	F
91 M	3/270	91	18	16/12/97	10/3/99	15	21	8	F
88 A	2/233	125	17	8/4/97	9/2/99	22	3	11	F
89 E	2/036	57	16	1/5/97	2/2/99	21	1	10	F
89 H	2/037	28	15	1/5/97	2/2/99	21	0	10	F
F 21	3/033	34	1	21/10/98	2/3/99	4	19	6	F
K 13	3/034	14	3	23/11/98	2/3/99	3	16	2	M
91 G	2/458	70	15	18/3/97	16/2/99	23	12	8	F
F 3	2/497	56	11	5/11/96	17/2/99	27	23	6	F
F 25	2/602	38	2	29/9/98	23/2/99	5	25	6	F
E 86	3/088	39	5	2/4/97	4/3/99	5	9	6	M
91 H	4/496	101	3	12/5/94	21/4/99	59	22	8	M
K 22	3/236	54	2	27/11/98	9/3/99	3	30	2	M
F 4	**2/496**	**97**	**17**	**23/11/98**	**17/2/99**	**3**	**14**	**6**	**F**
91 U	4/495	112	15	29/1/98	21/4/99	15	17	8	M
91 P	2/646	59	21	20/10/98	24/2/99	4	15	8	F
91 F	2/457	56	21	10/9/98	16/2/99	4	15	8	F
91 T	2/603	39	20	28/1/98	23/2/99	13	17	8	F
E94	**3/089**	**67**	**13**	**18/3/97**	**4/3/99**	**24**	**23**	**6**	**F**
K 23	3/237	31	1	22/10/98	9/3/99	5	27	2	M
89 C	2/647	62	19	10/9/98	24/2/99	5	20	10	F
F 11	**3/271**	**84**	**11**	**27/10/98**	**10/3/99**	**4**	**23**	**6**	**F**
91 N	3/428	135	12	28/1/98	16/3/99	7	12	8	M
90 A	3/429	116	15	19/3/97	16/3/99	24	13	9	M
91 K	3/487	77	21	9/9/98	18/3/99	6	14	8	M
91 V	3/488	101	8	3/12/97	18/3/99	16	19	8	M
91 B	3/610	92	10	2/12/97	23/3/99	16	13	8	M
E 93	2/334	43	13	3/9/97	11/2/99	17	16	8	F
88C	2/335	123	15	1/12/97	11/2/99	14	16	11	F
90 E	3/708	114	15	20/3/97	25/3/99	24	9	9	F
F 33	4/451	83					26	>4	F
F 30	4/452	69					24	<4	F
F 32	3/611	113					16	>4	F
F 35	3/707	73					4	>4	F
F 27	4/529	41					26	>4	F
F 26	4/530	32					8	>4	F

Evidence of lesions characteristic of decompression damage

Lesions consistent with previous spinal cord white matter infarcts (focal aggregation of fibres undergoing Wallerian degeneration or focal glial scars in white matter tracts) were found in three goats (with corresponding DERA codes in parentheses); 2/496 (F4) at the level of the fifth cervical spinal nerve, 3/271 (F11) at the level of the second to third lumbar spinal nerves and 3/089 (E94) at the second thoracic spinal nerve level (Fig. 6). The first two lesions were both bilateral, although not evenly sized, and involved dorsal columns, whereas the third was unilateral and situated in the ventrolateral area. The first two lesions were detected in both H&E stained and GFAP stained sections from the same level whereas the third was first detected on the GFAP stained section allowing subsequent location on the corresponding H&E stained section. The lesion in the first goat showed prominent focal Wallerian degeneration whereas in the second and third the lesions were both in the form of a glial scar (see Fig 4.). All these goats had a history of decompression illness (see Table 2) and comprised 18% of the total (17) within this group.

Further analysis revealed there was no correlation between the extent of Wallerian degeneration in longitudinal sections and the number of dives ($r_s = 0.279$; $P = 0.136$). There was a negative correlation between the extent of Wallerian degeneration and the time since the last dive ($r_s = 0.390$; $P = 0.03$). Notably, there was a significant correlation between the extent of Wallerian degeneration in longitudinal sections and the animals age ($r_s = 0.498$; $P = 0.005$). With regard to astrocytosis, there was a significant negative correlation between the animals age ($r_s = -0.651$; $P < 0.001$) and the number of dives ($r_s = -0.455$; $P = 0.012$) with the degree of astrocytosis. There was no correlation between the time from the last dive and the degree of astrocytosis ($r_s = -0.286$; $P = 0.125$).

Table 6: Spearman Rank Correlations performed on the data in Table 5.

Parameters compared	Correlation coefficient (r_s) and probability	Significant
W.D. (L.S.) and Astrocytosis (overall).	$r_s = -0.196$ ($P = 0.252$)	No
W.D. (L.S.) and No. of dives	$r_s = 0.279$ ($P = 0.136$)	No
W.D. (L.S.) and post-mortem interval (from last dive)	$r_s = 0.390$ ($P=0.03$)	Yes
W.D. (L.S.) and age	$r_s = 0.498$ ($P=0.005$)	Yes
Astrocytosis and age	$r_s = -0.651$ ($P= <0.001$)	Yes
Astrocytosis and post-mortem interval (from last dive)	$r_s = -0.286$ ($P= 0.125$)	No
Astrocytosis and no. of dives	$r_s = -0.455$ ($P = 0.012$)	Yes

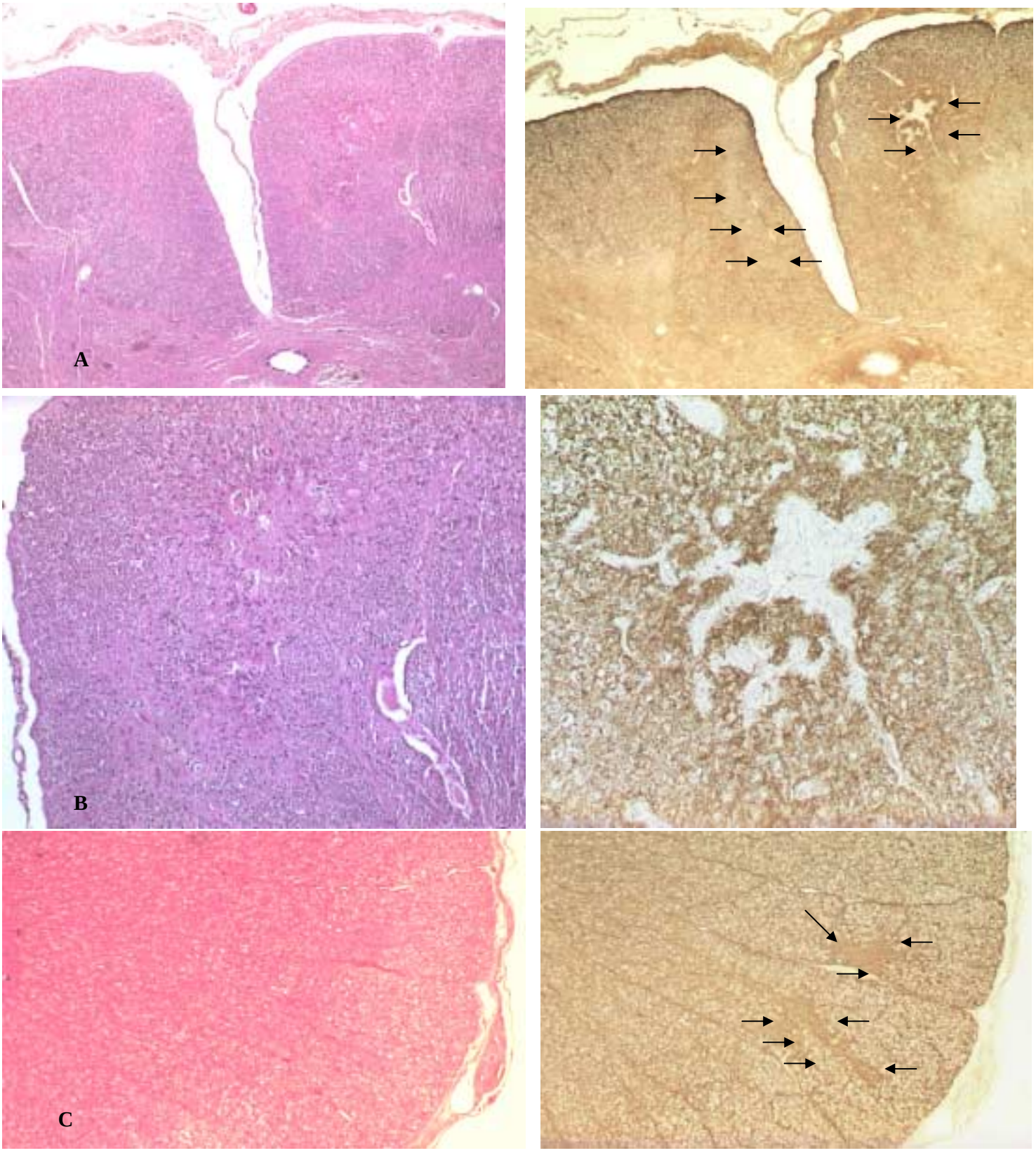

Figure 6: A shows low power appearance of severe glial scarring in both dorsal columns of goat F11 (arrows). B shows high power appearance of scar in left dorsal funiculus. C shows low power views of two smaller areas of glial scarring in the lateral column of goat E94.

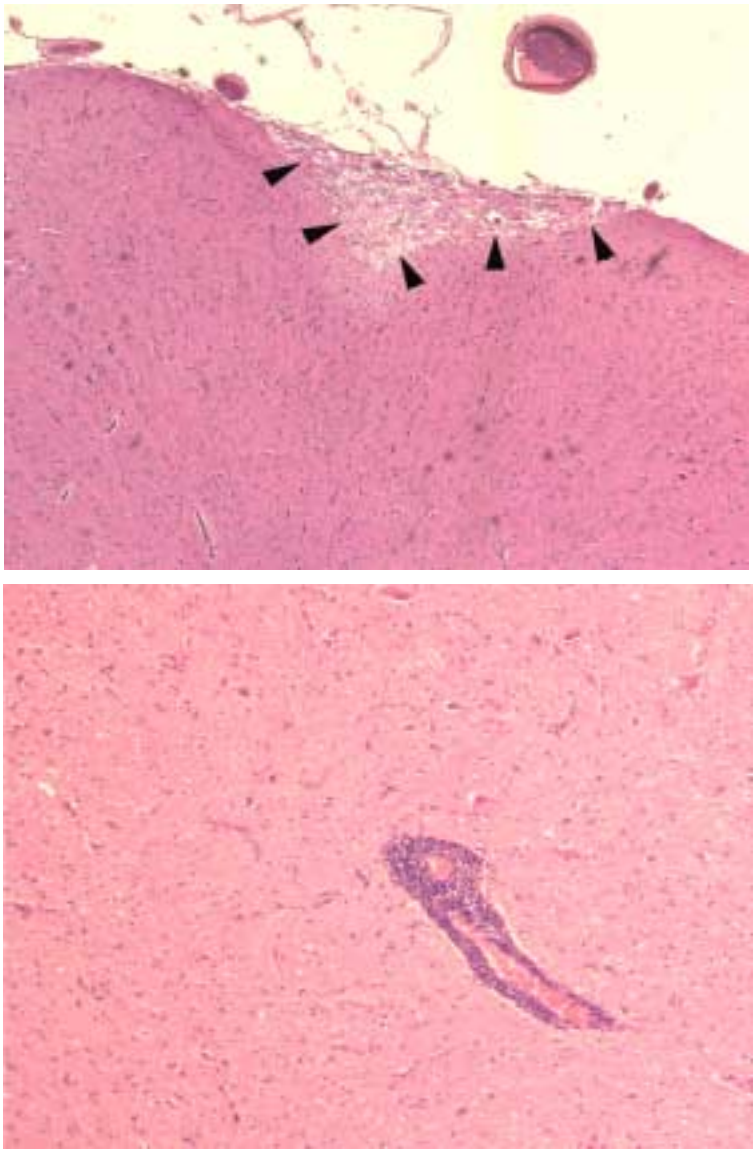

Figure 7: Examples of incidental lesions. A. . Focal cerebral lesion observed in goat F33. Arrows delineate a distinctly demarcated region of rarefaction of the neuropil.(X40 magnification). B. Perivascular cuff in the medulla.

Brain pathology
In two control goats (4/451 and 4/452) small focal superficial areas of cortical necrosis (see Fig. 5 A) were detected that showed no evidence of bilateral symmetry. Both lesions were only a few millimetres in size as they were contained within a single block and were not observed macroscopically. No other evidence of pathology was detected in the brain tissue examined from any of the exposed goats.

Other tissues examined
Although a limited range of pathological changes were detected in the sections from other organs no lesions were identified which could be related to diving exposure in the range of non-CNS tissues provided for each goat.

Discussion.

Pathological evidence of previous spinal cord injury consistent with that previously described in experimental animals exposed to decompression procedures (Palmer, *et al.*,1976; 1978; Blakemore and Palmer, 1982) was found in a small proportion (3/30) of the goats. It is notable that these three animals had all exhibited signs of decompression illness and the lesions were present at sites previously shown to be predilection sites for decompression pathology. Two of the goats had shown spinal decompression illness on one occasion and Type 1 decompression illness on a separate occasion (see Table 2), while the remaining goat had only one episode of Type 1 decompression illness. This was associated with its last hyperbaric exposure, approximately three months prior to euthanasia. The lesion detected in this animal was consistent with an episode of white matter infarction approximately three months prior to death. This finding of spinal infarction in an animal that did not show clinical signs associated with damage to the CNS reinforces the previous observation that spinal cord infarction can occur in the absence of clinical signs of Type 2 decompression sickness (Palmer *et al*., 1976).

Apart from the detection of pathological evidence of decompression illness in 3 of the symptomatic goats, we could not demonstrate significant difference in the severity of the pathology present in symptomatic and non-symptomatic exposed goats, with the possible exception of perivascular cuffing. This particular lesion is a non-specific inflammatory change and has not previously been described in association with decompression exposure as far as the authors are aware. It would therefore appear that there is a threshold for lesion development, and that this is superimposed on a background of diffuse axon degeneration and astrocytosis.

It was notable that there was significant increase in the severity of Wallerian degeneration in longitudinal section with age in the exposed animals. However, there was no correlation between this change and the number of hyperbaric exposures. In order to assess whether the severity of the pathological changes found was in any way related to repeated hyperbaric exposure requires a comparison to control animals that have not been exposed to decompression. We found there was no significant difference between the severity of the pathological changes examined between the exposed and control goats. However, there has to be a concern over both the low number and possible suitability of the animals submitted as controls.

Ideally the number of controls should be similar to the number of test subjects in each group to minimise losses in statistical power (Altman,1991). It is important to match for age, sex and husbandry, particularly as the scored parameters used in the present investigation (Wallerian degeneration and astrocytosis) are either known to be (Garcia-Segura *et al.,*1999), or may potentially be affected by all of these variables. Indeed, the most significant correlation we found in the hyperbaric animals was between animal age and extent of Wallerian degeneration in longitudinal section. In the current study the age of the control animals was uncertain, however, based on dentition 5 of the 6 control animals were over 4 years of age. All were female and had been maintained in different premises than the experimental animals, which were of both sexes. The control animals had significant amounts of Wallerian degeneration and this is known to be a change that is more frequently observed in older animals. In addition there was significant evidence of astrocytosis in three animals. This change can be associated with many causes, some of which are related to organic damage to the nervous system. The procurement of control animals was beyond our control since we were required to conduct our evaluation blind and it was only when the codes were broken that we became aware of the low number of control animals in the study. Because of the questionable value of the controls, as the data from the study stands, we cannot conclude that there is no evidence that repeated exposure to hyperbaric conditions leads to an increase in axon damage in the spinal cord over that which would be expected in the normal population. However, within the exposed animals there was no relationship between the number of dives and the incidence of Wallerian degeneration as might be expected if repeated exposure to decompression was associated with an accumulation of spinal cord damage. This agrees with the results of a study of spinal cords from 10 amateur and 10 professional divers that employed routine histopathology and a panel of immunohistochemical markers, including GFAP (Morke et al. 1994), which found no evidence of sub-acute or chronic changes in either white or grey matter that could be attributed to hyperbaric exposure.

Upregulation of GFAP expression is a well-recognised response of CNS tissue to injury and it was hoped that evaluation of GFAP stained sections would provide a sensitive method of evaluation. A visual comparison and scoring protocol was chosen since preliminary investigations into computer assisted methods of assessing staining intensity and distribution proved to be unreliable. This was due to the existence of a number of variables affecting intensity of staining which could not be controlled, such as time period of tissue fixation and uneven section thickness. The scoring scheme was validated by establishing that scores recorded independently by two observers were not significantly different. We found there was no positive correlation between severity of astrocytosis and the extent of Wallerian degeneration in any of the groups as might have been expected. In fact in the case of the group showing decompression illness there was a negative correlation, significant at the 5% level. Moreover, a similar negative correlation was found between astrocytosis and age and astrocytosis and number of dives when all animals were compared for these parameters. These results were unexpected. It is possible that immunohistochemical detection of GFAP upregulation is a short lived response in comparison to morphological evidence of axon damage as it is known that with long standing astrocytosis GFAP expression is down-regulated. There was no significant difference between control and exposed goats.

In contrast to the situation in man where a cerebral vasculopathy has been described in divers (Palmer *et al.*, 1992) no pathology was detected in the brain sections examined from exposed animals. However, two of the control animals exhibited focal brain lesions that would be consistent with small areas of cortical infarction. It is possible these could be related to head butting. The absence of detectable brain pathology is the exposed group was not unexpected since brain lesions in goats were not found in previous studies examining brain sections from animals showing extensive spinal cord pathology (Palmer *et al.*,1992).

Summary

A blinded detailed examination of formalin-fixed, paraffin-embedded CNS material stained by haematoxylin and eosin and GFAP immunohistochemistry found that lesions of decompression sickness are more likely to be found in animals that have experienced clinical signs of decompression sickness than those that have not. The study also illustrated that the extent of axon degeneration increased with age, however; we found no evidence for an accumulation of damage to spinal cord axons in animals exposed to repeated hyperbaric conditions over that found in non-exposed animals. These conclusions are drawn from examination of post-mortem material derived from 30 exposed goats and 6 control animals. The low number and suitability of the control animals does not allow us to state categorically whether this age related incidence of axon damage in the animals exposed to hyperbaric conditions was greater than would be expected as a normal ageing phenomenon.

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Glossary

Astrocyte: The glial cell responsible for homeostasis of CNS environment. Reacts by hypertrophy (astrocytosis - increased expression of GFAP) to alterations in CNS environment of cell damage

Axon: Cell process of neuron that conducts electrical impulses.

Caudal: Refers to the aspect of an area closest to the tail (opposite to cranial/rostral).

Chokes: A syndrome of respiratory symptoms occurring amongst sufferers of decompression illness.

Dorsal: Refers to the aspect of an area closest to the back bone.

Dyspnoea: Abnormal breathing.

Funiculus: A clearly defined white matter tract.

Glia: All cells within the normal central nervous system excluding neurones vascular endothelium, connective tissue mesenchymal cells.

Glial acidic fibrillary protein (GFAP): The protein constituent of astrocyte intermediate filaments.

Glial scar: Focus of disruption of CNS tissue characterised by an accumulations of astrocyte processes.

Grey matter: The tissue of the central nervous system occupied predominantly by nerve cell bodies.

Immunohistochemistry: The technique of using appropriate antibodies to impart colour to cells expressing specific antibody binding sites**.**

Infarct: A discrete area of tissue loss resulting from occlusion of its blood supply.

Median: In a plane passing through the midline of a structure and which bisects it dorso-ventrally into equally sized lateral components.

Myelophage: An apoptotic macrophages found within myelin ovoids – a hallmark of Wallerian degeneration.

Necropsy: Perform a post-mortem examination.

Neuropil: Dense meshwork of cytoplasmic processes from nerve cells (axons and dendrites) and glial cells forming the tissue separating neuronal cell bodies.

Paresis: Weakness.

Perivascular cuffing: The accumulation of lymphocytes and macrophages around blood vessels.

Pruritus: Cutaneous irritation (itching) most frequently relieved by scratching.

Quadriplegia: Paralysis of four limbs.

Rostral: Refers to an aspect of an area of the brain closest to the nose.

Saggital: In a plane parallel to the median plane.

Ventral: The aspect of an area away from the back bone

Wallerian degeneration: **W**hen axons are cut, the axon distal to the point of transection becomes separated from the neuronal cell body and degenerates. Wallerian degeneration is the term used to describe the pathology that is associated with axon degeneration.

White matter: Areas of the CNS composed of myelinated axons and few if any neuronal cell bodies

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