

Pharmacokinetic behaviour of ACP gel, an autocrosslinked hyaluronan derivative, after intraperitoneal administration

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Abstract

Autocrosslinked polysaccharide (ACP) gel is a fully biocompatible cross-linked derivative of hyaluronic acid, which has prolonged *in vivo* residence time and improved mechanical properties with respect to native hyaluronan for use in various surgical applications. The objective of this study was to assess the pharmacokinetic behaviour of ACP gel in dogs after intraperitoneal administration. Seven beagle dogs received intraperitoneal injections of tritium-labelled ACP gel. Blood samples were taken, and urine and faeces were collected until sacrifice, scheduled at various time points from 3 to 192 h after administration. Organs were removed from the animals at autopsy. Bodily fluid and organ samples were analysed for total and non-volatile radioactivity.

Non-volatile radioactivity slowly appeared in plasma, with a median T_{\max} of 12 h, and then declined with a mean half-life of 69 h. Total radioactivity in plasma peaked later and declined more slowly, consistent with the formation of tritiated water. Little non-volatile radioactivity was found in any organs except the liver, where about 16% of the dose was present 72 h after administration, and the intestines, where the presence of radioactivity was probably due to a retention effect. A minor amount of non-volatile radioactivity was also found in the bone marrow.

In summary, ACP gel administered into the peritoneal cavity is removed slowly by active initial catabolism at the injection site, and is then catabolised by well described physiological pathway of hyaluronan degradation with final release of simple molecules such as CO_2 and H_2O . Given its *in vivo* residence time, ACP gel may be considered an ideal implantable surgical device.

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1. Introduction

Hyaluronic acid, or hyaluronan (HA), is a water-soluble polysaccharide that is widely distributed throughout a variety of tissues in humans and other animals [1]. It consists of multiple disaccharide units of glucuronic acid and *N*-acetylglucosamine, bound together by a β 1-3-type glucoside bond. It has a high molecular weight, usually of the order of millions

of Daltons, and solutions of HA have interesting visco-elastic properties [1] which have led to investigation in applications of HA in surgical procedures, for example in visco-supplementation, ocular surgery and prevention of postsurgical adhesions [2]. A substance that could effectively prevent adhesions would be an important weapon in the surgeon's armamentarium, as postsurgical adhesions account for substantial morbidity, such as intestinal obstruction and infertility [2,3]. The healthcare costs of postsurgical adhesions are also substantial [2,4].

HA is an attractive substance for use in surgical procedures because it occurs naturally in the human

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body, and is therefore fully biocompatible. However, HA is not an ideal substance for all procedures, due to its limited residence time when applied to a surgical site. It quickly enters the systemic circulation via the lymph, and is then cleared rapidly by catabolic pathways [5]. Attempts to use hyaluronan for preventing postsurgical adhesions have therefore been met with mixed success [6,7].

In an attempt to circumvent the disadvantages of HA, various chemically modified derivatives of HA have been developed [8]. One such derivative is autocrosslinked polysaccharide (ACP). It is formed by crosslinking hyaluronan, via direct formation of covalent ester bonds between hydroxyl and carboxyl groups of the hyaluronan molecule [9]. Such ester bonds may be either intramolecular (within a single hyaluronan molecule) or intermolecular (linking two hyaluronan molecules together). The advantage of this direct formation of ester linkages over alternative methods is that no foreign substances are introduced into the molecule, thus catabolism of ACP leads only to hyaluronan. ACP can be prepared with various degrees of crosslinking, which allows tailoring of the viscosity properties of ACP gels [9]. ACP gels have proven to be effective in preventing postsurgical adhesions in animal models [3,10,11] and in the clinical practice [12–14].

Given the use of ACP gel as in implantable surgical device, it is important to understand how it behaves in vivo. The present study was therefore conducted to investigate the pharmacokinetics of ACP gel after intraperitoneal administration in dogs and to identify, if possible, its degradation pathway.

2. Materials and methods

2.1. Study design

The study consisted of two phases. The first was a pilot phase, in which radio-labelled ACP gel was administered to one animal. Blood samples, urine, and faeces were collected up to 72 after dosing, at which time the animal was killed for analysis of radioactivity in organs. This pilot phase served to define suitable sampling times for the main phase of the study. In the second phase, a further six animals received radio-labelled ACP gel. One animal was killed at each of the following times after dosing: 3, 12, 24, 72, 120 and 192 h. Up to the time the animals were killed, blood samples, faeces, and urine were collected. Distribution of radioactivity in the tissues and organs were determined at autopsy. This animal study was done in a experimental facility qualified for the conduction of pharmacokinetics studies and GLP-certified.

2.2. Test substance

ACP gel is an auto-cross-linked HA derivative in the form of highly viscous gel. The polymer is obtained through a cross-linking process that results in condensation and, therefore, does not introduce foreign bridge molecules. The chemical functions that contribute to the cross-linking process are the carboxyl groups and the hydroxyls, preferably the primary ones belonging to glucosamine. The reaction is begun by activating about 5% of the carboxyl groups using a suitably spiked condensing agent followed by nucleophilic attack by the hydroxyl groups. An ester-type bond is thus created which may involve the chain of hyaluronic acid itself (intramolecular crosslink) as well as other chains. Modification of the structure creates a complex web of polymer chains that increase the molecular weight practically to an infinite degree, and this influences the viscoelastic properties of the product when it absorbs water. Large quantities of the solvent are able to penetrate between the molecular mesh, helping the structure to swell and thus giving rise to a gel.

For the conduction of this study, tritiated ACP gel (specific activity 1.11 $\mu\text{Ci}/\text{mg}$) was supplied by Fidia Advanced Biopolymers in pre-formulated glass syringes, each of which contained 360 mg ACP gel (400 μCi). The preparation of the radioactive material involved three different steps. The first step was a partial de-acetylation of the starting hyaluronic acid sodium salt, which consisted in reacting HA in a solution of hydrated hydrazine in the presence of hydrazine sulphate. Operating at temperatures not exceeding 50–55 °C overnight, it was possible to obtain 8% de-*N*-acetylation, without significantly influencing the distribution or the mean molecular weight value (about 180 kDa). The intermediate product was then separated from the reaction mixture by alcoholic precipitation.

Subsequently, the HA-de-acetylate underwent re-acetylation with tritiated acetic anhydride (500 mCi/mol) in a mildly basic aqueous solution. The intermediate product was separated by adding ethanol. In the last step of the process, the tritiated HA in sodium form was percolated through a column previously filled with resin activated for ion exchange with tetrabutylammonium (TBA). The salt of HA-tritiated TBA was then solubilised in *N*-methyl pyrrolidone and reacted in the presence of condensing agent chloro-methylpyridine iodide (CMPJ). The crosslinked product was then separated from the reaction mixture by precipitating it with ethanol in the presence of a saturated sodium chloride solution. Once the raw product had been dissolved in water, it was dialysed to eliminate any contaminants from the process. At this stage, the radioactivity of the eluate was monitored, stopping the procedure only when the reading reached approximately background values: in this way it was possible to be

reasonably sure that all the radioactivity of the product was bound to the tritium belonging to the acetic group of the glucosamine residue. Lastly, the product was freeze-dried before being hydrated and sterilised.

3. Animal experiment

Male and female beagle dogs, approximately 8 months old (weight range 9.6–13.6 kg), were used in the study. Animals were acclimatised on the experimental site for at least 2 weeks before dosing, during which time their health was monitored at least daily. During the study, animals were individually housed in stainless steel metabolism cages equipped for quantitative collection of urine and faeces. Throughout the acclimatisation and study periods, the animals were fed a standard pelleted dog diet, and allowed tap water *ad libitum*. Before administration of the test substance, a needle attached to a syringe containing saline was introduced into the abdominal cavity under sterile conditions, and then aspirated to ensure that the needle had not penetrated any organ. The needle was then removed from the saline syringe and attached to a syringe containing ACP gel, which was injected over approximately one minute.

3.1. Sampling of bodily fluids, excreta, tissues, and organs

Blood samples were collected at various times after administration until the animals were killed. Part of the blood samples were retained for analysis of whole blood, and the remainder was centrifuged for analysis of plasma. Urine and faeces were collected quantitatively from the metabolism cages at 24-h intervals. In addition, urine was collected in the first 8 h after dosing. At the end of each faecal collection, the metabolism cages were thoroughly washed with water to account for any residual radioactivity. At each of the designated sacrifice times, one animal was euthanised by an overdose of barbiturate. At autopsy, the following organs were removed for analysis of radioactivity: adrenals, blood, bone mineral, bone marrow, brain, eyes, fat (white), heart, kidneys, large intestine (and contents), liver, lungs, muscle, ovaries (females), plasma, prostate (males), skin, small intestine (and contents), spleen, stomach (and contents), testes (males), thyroid, injection site, uterus (females). Radioactivity in the remaining carcass was also assayed.

3.2. Analytical methods (sample preparation, radioactivity counting)

All samples were analysed in at least duplicate. Liquid samples were mixed with approximately 10 ml of Ready Safe™ liquid scintillator. Plasma and urine samples for

non-volatile radioactivity analysis were weighed into scintillation vials and allowed to dry overnight, resuspended in 1 ml of water and then mixed with Ready Safe™ liquid scintillant prior to counting to determine the proportion of volatile radioactivity lost. Tap water was added to each faeces and carcass samples before they were homogenized. Carcass samples were minced prior to homogenisation. Aliquots of faeces, carcass homogenates and tissue samples were weighed into combusto-cones and combusted immediately using a Packard automatic sample oxidiser to determine total radioactivity. Tissue, whole blood and faeces samples were weighed into combusto-cones and left to dry at least overnight prior to combustion to determine non-volatile radioactivity levels. Water and Monophase®S were used as absorbent and scintillator respectively.

Plasma, urine, cage wash and dose estimate samples were counted directly in Ready Safe™ liquid scintillator. Faeces, carcass, blood and tissue samples, as described above, were combusted in a Packard automatic sample oxidiser. Samples were counted in a model LS6000IC liquid scintillator counter using an H-number method for quench correction. Quench correction was checked using quenched radioactive, reference standards. Samples were counted to a Sigma 2 counting error of 0.5% of for a maximum of 10 mins. Appropriate liquid scintillation blanks were used to establish background levels of radioactivity.

3.3. Statistical methods

No statistical hypotheses were tested; the data are presented descriptively. Pharmacokinetic parameters were calculated from the radioactivity data in plasma and whole blood using non-compartmental analysis. Half-life was calculated by non-linear regression of the terminal portion of the concentration-time curve, in which the data were iteratively reweighed by the squared reciprocal of the predicted plasma drug concentrations. Half-life was accepted as valid provided that the descending portion of the concentration-time curve had been reached, at least 3 points were available for the regression, and the relative standard error of the estimate of the elimination rate constant was less than 40%. If those criteria were not met, then half-life and the parameters that are calculated from it are not reported. T_{max} and C_{max} are not reported if the concentration was still ascending at the final sampling time.

4. Results

4.1. Radioactivity in plasma and blood

The radioactivity levels detected in plasma after intraperitoneal administration of ³H-ACP gel were low

at all sampling times. The plasma radioactivity consisted of two fractions: a non-volatile fraction that reached a peak at around 12–24 h after dosing and a volatile fraction (tritiated water) that was detectable in plasma after 12–24 h, corresponding to a peak of non-volatile radioactivity. Non-volatile radioactivity in plasma declined slowly, with a mean half-life of almost 3 days (Table 1). The non-volatile fraction in whole blood followed a similar pattern to that seen in plasma (Table 1, Fig. 1). The plasma radioactivity levels for the last two dogs to be killed are shown in Fig. 2, which shows that plasma non-volatile radioactivity was still at measurable levels even up to 192 h after dosing.

Total radioactivity in plasma peaked later than the non-volatile fraction, and declined more slowly (Fig. 2). This was due to the presence of volatile radioactivity that has a peak 72 h after the administration; at peak the volatile fraction constituted 83–91% of total plasma radioactivity. The plasma decay of total radioactivity was slower than that of the non-volatile fraction but its pharmacokinetic behaviour was difficult to assess, as 5 of the 7 dogs had been killed before plasma total radioactivity has reached a maximum.

4.2. Excretion of radioactivity

Urinary excretion of radioactivity is summarised in Table 2, and was consistent with the time course of the plasma levels. Non-volatile radioactivity was the main fraction until 72 h after dosing; thereafter, the non-volatile fraction was replaced by the volatile fraction. Urine appeared to be the major route of excretion: less than 5% of administered radioactivity was collected in faeces by 120 h after dosing.

4.3. Radioactivity in tissues and organs

A substantial amount of the administered radioactivity remained within the animals throughout the study, most of which was in the remaining carcass after the organs had been removed. The amount of total radioactivity in the carcass was equivalent to about 60% of the total dose at 3 and 12 h after dosing, and declined to about 20% by 192 h. There was little penetration of non-volatile radioactivity into specific organs, with the exception of the liver and gastrointestinal tract.

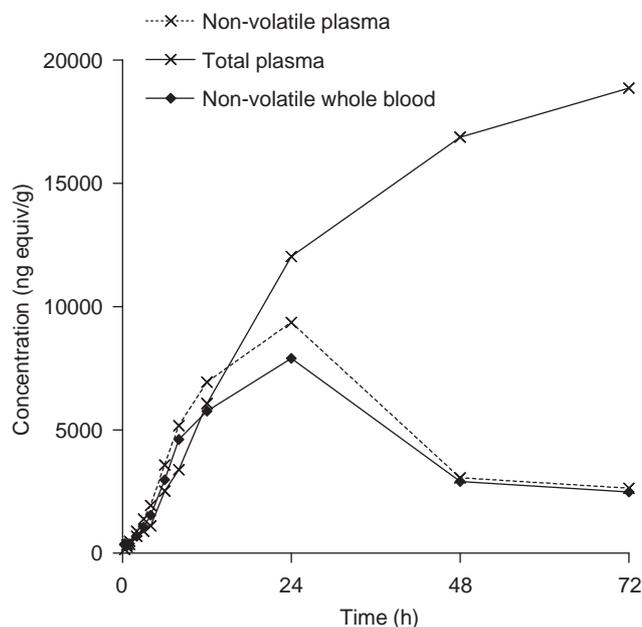


Fig. 1. Mean radioactivity concentrations in blood and plasma up to 72 h.

Table 1

Pharmacokinetic parameters of radioactivity in plasma and whole blood after intraperitoneal administration of [^3H]-ACP gel

	N	Mean	SD	Median	Min	Max
<i>Non-volatile radioactivity in plasma</i>						
T_{\max} (h)	5	15.2	8.20	12.0	8.00	24.0
C_{\max} ($\mu\text{g equiv/g}$)	5	11.9	6.31	10.0	7.26	22.6
AUC_{0-24} ($\mu\text{g equiv/g h}$)	5	130	38.2	128	73.6	169
$\text{AUC}_{0-\infty}$ ($\mu\text{g equiv/g h}$)	4	701	280	674	418	1040
Elimination half-life (h)	4	69.2	36.7	61.6	35.3	118
<i>Total radioactivity in plasma</i>						
T_{\max} (h)	2	72.0	0.0	72.0	72.0	72.0
C_{\max} ($\mu\text{g equiv/g}$)	2	20.8	8.28	20.8	15.0	26.7
AUC_{0-24} ($\mu\text{g equiv/g h}$)	5	137	45.5	118	81.5	185
$\text{AUC}_{0-\infty}$ ($\mu\text{g equiv/g h}$)	2	5535	3514	5535	3050	8020
Elimination half-life (h)	2	137	46.3	137	105	170
<i>Non-volatile radioactivity in whole blood</i>						
T_{\max} (h)	5	16.0	7.48	12.0	8.00	24.0
C_{\max} ($\mu\text{g equiv/g}$)	5	10.6	5.63	10.0	5.53	19.5
AUC_{0-24} ($\mu\text{g equiv/g h}$)	5	110	31.8	123	55.9	135
$\text{AUC}_{0-\infty}$ ($\mu\text{g equiv/g h}$)	3	615	335	478	371	997
Elimination half-life (h)	3	75.6	48.2	52.6	43.2	131

Within the gastrointestinal tract, the largest amounts of both volatile and total radioactivity were found in the small intestine. The radioactivity counts in the liver and small intestines are shown in Figs. 3 and 4. The time course of radioactivity in the two organs were different, with the highest radioactivity concentrations in the small intestine at early time points, whereas concentrations in the liver rose to a maximum at 72 h after dosing, reaching the 15.3% of the dose administered. Moreover, a small percentage, ranging from 1.4% to 4.9% of the dose administered, was found in the bone marrow, assuming an overall weight of this tissue being 8.5% of the total body weight.

5. Discussion

ACP is a cross-linked derivative of hyaluronan. From a chemical point of view, ACP is an inner ester

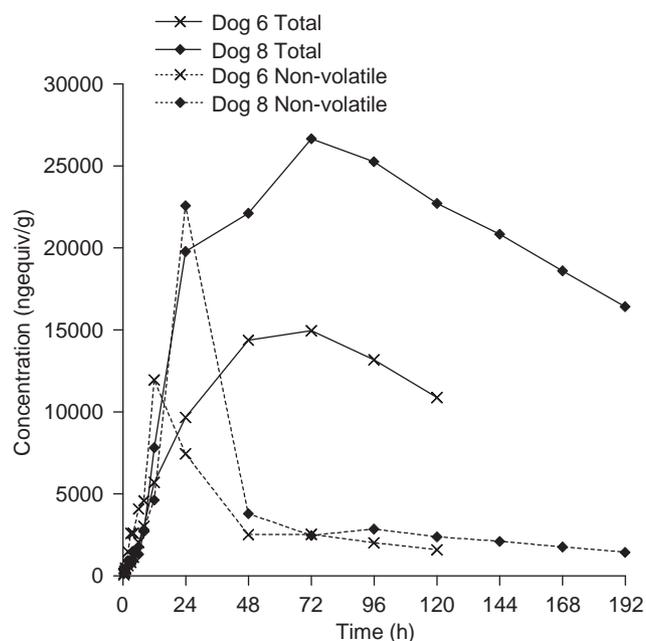


Fig. 2. Individual radioactivity concentrations in plasma in dogs killed after 120 and 192 h.

Table 2
Urinary radioactivity excretion (% of dose)

	Total radioactivity			Non-volatile radioactivity		
	N	Mean	SD	N	Mean	SD
8–24 h	4	12.0	2.1	4	12.8	4.0
24–48 h	4	5.2	2.0	3	7.9	3.8
48–72 h	4	3.8	2.0	1	4.4	—
96–120 h	2	3.1	0.7	1	1.5	—

Total radioactivity should always be greater than or equal to non-volatile radioactivity: that this is not so at early timepoints is due to limitations of the assay method.

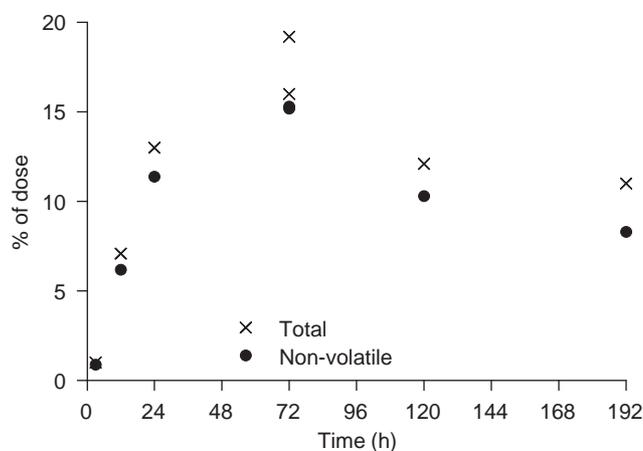


Fig. 3. Radioactivity in liver.

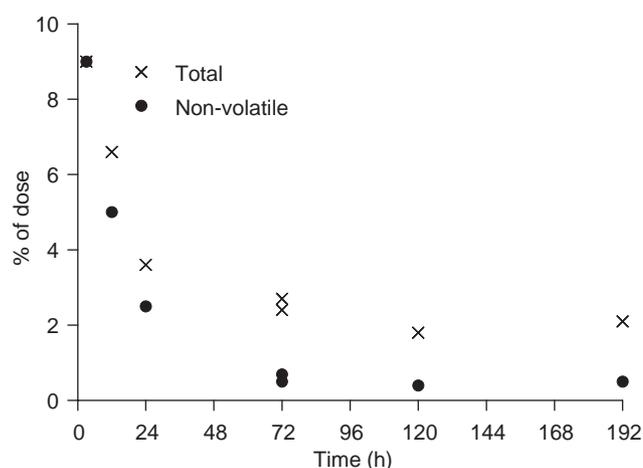


Fig. 4. Radioactivity in small intestine.

constituted by a carboxylic residue of hyaluronic acid with a hydroxyl residue of the polysaccharide chain. This chemical transformation leads to a structural change in the hyaluronan molecule by which the ACP compound becomes insoluble in water or aqueous systems. ACP forms a gel in the presence of hydrophilic matrices, thus giving rise to a heterogeneous system. Consequently, the product cannot be absorbed in an unmodified form. However, within the organism, the compound may undergo both chemical and enzymatic degradation by esterases, enzymes that are ubiquitous in all vertebrates. Cleavage of the inner ester bond most likely reconstitutes the starting molecule, hyaluronan. Because of hyaluronan's large molecular size, it cannot easily pass through biological membranes, so its catabolism occurs predominantly in situ by the action of hyaluronidase, another enzyme ubiquitously distributed in the organism.

It is necessary to bear these generalisations in mind when interpreting experimental findings. Radioactive

labelling was the only way of tracing the product's fate, even roughly. A strength of radioactive labelling is that it allows the location of the test substance to be determined precisely after intraperitoneal administration, but a disadvantage is that it does not provide information about the nature of any metabolic products. Some hypotheses are however possible:

- (1) The non-volatile plasma radioactivity consists of products of the catabolism of first ACP and then hyaluronan. A first, initial catabolism of ACP gel occurs at the administration site (peritoneum) with formation of low-molecular weight fragments. These are taken up by the peritoneal vessels and reach the liver, which is the major site of circulating HA uptake and degradation, as is largely established [19]. The presence of low-molecular weight residues at the early sampling times reflects therefore initial catabolism of ACP gel, and perhaps in little part also the absorption of residues present in the material as by-products entrapped in the polymer network during the synthesis of the radioactive product.
- (2) The volatile plasma radioactivity is an indicator of terminal metabolism of the compound, which may be both local (at peritoneal membrane level) and hepatic. Indeed, all the molecular fragments that pass through the peritoneum and follow the normal metabolic pattern of polysaccharides are conveyed to the liver, as mentioned above.

The time-course of plasma radioactivity of the two volatile and non-volatile fractions supports these hypotheses. The non-volatile fraction was mainly present at the early test times and dropped substantially after 12–24 h. On the other hand, volatile radioactivity began to make an appearance later, peaking after about 72 h. By extrapolation, it is possible to deduce that ACP gel remained in the peritoneal cavity for at least 24–48 h, and probably even longer. The tissue distribution data also support this interpretation. The only organ where accumulation occurred is the liver, where about 16% of the dose was present 72 h after administration. Although substantial radioactivity was also present in the intestines, the different time course of radioactivity at that site suggests a retention effect rather than active uptake. Findings obtained for the bone marrow suggest that this was a minor uptake organ for the radioactive tracer, in agreement with recently published data on biodistribution of hyaluronic acid [15], however the administration route in that study was different (intravenous). Most of the radioactive dose injected was found in the carcass, confirming ACP's slow removal from the administration site.

The slow appearance of volatile radioactivity is consistent with metabolic degradation of ACP gel to form tritiated water. The half-life of the disappearance

of the total radioactivity from plasma was several days, which is in agreement with the half-life of labelled water in other mammalian species [16–18].

6. Conclusions

ACP gel administered into the peritoneal cavity was removed very slowly by active initial catabolism at the injection site and the only products of catabolism are CO₂ and H₂O, confirming the same metabolic pathway of native unmodified hyaluronan. This characteristic, together with the muco-adhesive properties of HA and its derivatives, suggests a possible use of the product in a wide range of surgical applications including ophthalmic surgery, aesthetic surgery to augment dermal and perimucular connective tissue, visco-surgical tool in orthopaedic surgery. More recently ACP gel, in different concentrations, has been successfully used as visco-elastic biocompatible “barrier substance” to prevent post-surgical adhesions and excessive scar formation in abdominal-pelvic area, tendon and peripheral nerve surgery of the hand, and orthopaedic surgery. The product after administration in the anatomical sites create a HA-rich environment modulating the wound healing process of the peritoneum and restoring the gliding function of the tendon and peripheral nerve structures.

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