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Quantification of neuropeptides (calcitonin gene-related peptide, substance P, neurokinin A, neuropeptide Y and vasoactive intestinal polypeptide) expressed in healthy and inflamed human dental pulp

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Abstract

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Aim To quantify the expression of calcitonin gene-related peptide (CGRP), substance P (SP), neurokinin A (NKA), neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP) in healthy and inflamed human dental pulp tissue.

Methodology Six pulp samples were obtained from teeth having a clinical diagnosis of acute irreversible pulpitis. Another 12 pulp samples were obtained from premolars where extraction was indicated for orthodontic purposes. In six of these premolar teeth inflammation was induced by mechanical pulp exposure prior to sample collection. All samples were processed and ^{125}I-labelled; neuropeptides were quantified by competition assays. ANOVA and Mann–Whitney's (*post hoc*) tests were used to establish statistically significant differences between the groups.

Results Expression of five neuropeptides was found in all human pulp samples. Statistical analysis revealed a significantly higher ($P < 0.05$) expression of CGRP, SP, NKA and NPY in both inflammatory conditions compared with healthy pulp control values. VIP expression remained stable during the inflammatory conditions.

Conclusion Expression of CGRP, SP and NKA released from C-fibres and NPY released from sympathetic fibres is significantly higher in the inflamed human pulp compared with healthy pulp. Expression of VIP released from parasympathetic fibres is not increased during the inflammatory conditions of human dental pulp.

Keywords: human dental pulp, neurogenic inflammation, neuropeptides, radioimmunoassay.

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Introduction

Dental pulp is a soft mesenchymal tissue densely innervated by afferent (sensory) fibres originating from the trigeminal ganglion (Wakisaka 1990) and sympathetic fibres originating from the cervical sympathetic ganglia (Avery *et al.* 1980). There are controversies regarding the parasympathetic innervation of the human pulp by fibres originating in the otic ganglion (Segade *et al.* 1987). This complexity in pulp innervation becomes evident when studying the interactions between nervous fibres and pulp physiology and pathology (Olgart 1996).

Dental pulp inflammation is a complex process involving a wide variety of nervous and vascular reactions, which are key components of the neurogenic inflammation that could lead to pulp necrosis (Kim 1990).

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Neuropeptides play an active role during neurogenic inflammation of the pulp, controlling its blood flow and regulating later stages of inflammation and repair processes (Olgart 1996). These neuropeptides include calcitonin gene-related peptide (CGRP), substance P (SP), neurokinin A (NKA), neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP) among others (Casasco *et al.* 1990, Wakisaka 1990, Goodis & Saeki 1997).

Sensory-derived neuropeptides, such as CGRP, SP and NKA, are produced in trigeminal cell bodies and transported via axonal transport to nerve terminals in the pulp where they are stored (Gazelius *et al.* 1987, Awawdeh *et al.* 2002). These nerve terminals are mainly C-type fibres that are closely related to pulp microcirculation, although some free nerve endings are also observed (Wakisaka 1990).

It has been demonstrated that CGRP and SP interacts with mastocytes, inducing the release of histamine and thereby causing elevated vascular permeability and increased blood pressure (Hargreaves *et al.* 1994). SP and NKA also interacts with other inflammatory cells such as macrophages and lymphocytes, altering its functions and inducing these cells to release inflammation mediators such as cytokines, prostaglandins and thromboxanes having a direct effect on the inflammatory process (Patel *et al.* 2003, Park *et al.* 2004).

Calcitonin gene-related peptide exerts stimulatory effects on the growth of pulpal cells, such as fibroblast and odontoblast-like cells (Trantor *et al.* 1995). It also increases the *in vitro* expression of bone morphogenetic protein (BMP) – 2 transcripts in human pulp cells, leading to odontoblast-like cell differentiation (Calland *et al.* 1997). Release of CGRP plays an important role in the clinical inflammatory condition of acute irreversible pulpitis (Caviedes-Bucheli *et al.* 2004a, 2005b).

Neuropeptide Y is a sympathetically derived neurotransmitter that is co-localized in terminals with noradrenaline (Uddman *et al.* 1998). Sympathetic fibres terminate as free nerve endings and are predominantly confined to blood vessels in the root and midcoronal region of the pulp (Avery *et al.* 1980, Rodd & Boissonade 2002). NPY was found to be a potent inhibitor of vasodilation induced by acetylcholine and SP, playing an important role in sympathetic vasoregulation (Fallgren *et al.* 1989). Administration of NPY to dental pulp reduces pulpal blood flow via vasoconstriction similar in magnitude to that produced by electrical stimulation of the sympathetic fibres that innervate the pulp (Kim *et al.* 1996).

The parasympathetic nervous system does not appear to have a dominant role in regulating pulpal blood flow as the sympathetic nervous system (Olgart 1996), although some studies have demonstrated the presence of parasympathetic fibres in dental pulp (Segade *et al.* 1987, Wakisaka *et al.* 1987). VIP is a parasympathetic-derived neurotransmitter that is co-localized in terminals with acetylcholine and increases pulpal blood flow (Uddman *et al.* 1980).

The study of these neuropeptides and understanding their role in pulp neurophysiology are important in developing therapies to control neurogenic inflammation of the pulp. The aim of this study was to use a radioimmunoassay for quantifying the expression of these neuropeptides in healthy and inflamed human dental pulp.

Materials and methods

A descriptive comparative study was performed according to Colombian Ministry of Health recommendations regarding ethical issues in research involving human tissues. Written informed consent was obtained from each patient participating in the study. Pulp samples were obtained from 18 different non-smoking human donors (19–40 year old; $n = 6$ per group): pulps from teeth having a clinical diagnosis of acute irreversible pulpitis (experimental group), pulps with induced inflammation (positive control group) and clinically normal pulp tissue (negative control group).

Six pulp samples were obtained from posterior teeth having a clinical diagnosis of acute irreversible pulpitis. These patients were suffering moderate to severe spontaneous pain of approximately 24-h duration. None of them were taking anti-inflammatory drugs. Teeth were anaesthetized (1.8 mL of 4% prilocaine by infiltration injection in the maxillary teeth and by inferior alveolar nerve block injection for mandibular teeth) and isolated with a rubber dam. Shortly after, cavity access was completed and pulp tissue was extracted with a sterile barbed broach.

Another 12 pulp samples were obtained from premolars extracted for orthodontic purposes. In six of these teeth, inflammation was induced prior to pulp collection. The remainder were used to establish normal neuropeptide expression. For the induced inflammation group, teeth were anaesthetised and isolated as described before. The inflammatory process was generated by mechanical exposure of the pulp using a no. 1 round carbide bur in a high-speed handpiece without irrigation. After a period of 10 min the pulp tissue was extracted using a sterile barbed broach.

For the group of healthy pulps the teeth were anaesthetized and extracted. Immediately after extraction teeth were washed with 5.25% sodium hypochlorite to eliminate remnants of periodontal ligament that could contaminate the pulp sample. The teeth were then sectioned using a cylindrical diamond bur in a high-speed handpiece irrigated with saline solution. Pulp tissue was obtained using a sterile endodontic excavator.

All of the pulp samples were placed into a pre-weighed Eppendorf tube with 4% paraformaldehyde and kept frozen at −70 °C until use. After all the samples had been obtained, a previously recommended neuropeptide extraction methodology was followed (Awawdeh *et al.* 1999). Eppendorf tubes were weighed and each pulp weight was calculated. Then 250 μL of 0.5 M acetic acid with protease inhibitors was added and brought to boiling in a water bath for 10 min. The tubes were centrifuged at 3500 rpm (2000 g) for 20 min per 4 °C (GS-6KR Centrifuge, Beckman, Fullerton, CA, USA) and each supernatant was collected and transferred to another tube.

Radioimmunoanalysis

Neuropeptide expression was determined by competition binding assays using Radioimmunoanalysis (RIA) kits from Phoenix Peptide Pharmaceutical (Belmont, CA, USA): Human CGRP RIA-kit (ref. RK-015-02), Human SP RIA-kit (ref. RK-061-05), Human NKA RIA-kit (ref. RK-061-05), Human NPY RIA-kit (ref. RK-049-03) and Human VIP RIA-kit (ref. RK-064-16). Each kit consists of standard and ^{125}I-labelled neuropeptide and antiserum-neuropeptide.

The assay system comprised 100 μL of antiserum, 100 μL radioactive tracer and 100 μL of different standard neuropeptide concentrations (1–128 pg μL^{-1}) or 100 μL pulp tissue extracts. All samples were assayed in duplicate and the mean values calculated. Following 24-h incubation, the suspensions were spun at 5000 rpm (4000 g) for 1 h (Beckman) to precipitate the bound fractions. The supernatants were decanted and pellet radioactivity was read on a Gamma Counter (Gamma Assay LS 5500; Beckman). Scatchard analysis of the binding data assessed the amount of neuropeptide present in every sample.

Statistical analysis

Values are presented as neuropeptide amount in pg mg^{-1} of wet pulp tissue. Mean and maximum/minimum values are presented for each neuropeptide in each group. ANOVA test was performed to establish statistically significant differences ($P < 0.05$) between the groups. Mann–Whitney's *post hoc* comparisons between groups were also performed.

Results

Expression of five neuropeptides was found in all human pulp samples. Healthy pulps showed the lowest expression for all neuropeptides, followed by the induced inflammation group. Highest neuropeptide expression was found in the acute irreversible pulpitis group (Fig. 1).

Differences between groups were statistically significant for all neuropeptides except VIP. ANOVA test showed statistically significant differences between the groups ($P < 0.05$). Mann–Whitney's *post hoc* tests showed statistically significant differences between experimental and both control groups ($P < 0.05$). Differences between negative and positive control groups were also significant ($P < 0.05$). Results for each neuropeptide are presented in Tables 1–5.

Discussion

This study compared the concentrations of CGRP, SP, NKA, NPY and VIP in healthy and inflamed human pulp tissue. The five neuropeptides were identified in all human pulp samples, whether inflamed or not, but having a significantly higher expression in pulps from teeth diagnosed with acute irreversible pulpitis, except for VIP which remained practically unaltered in the three groups evaluated.

The increased levels of CGRP, SP, NKA and NPY in the inflamed pulp, suggest that neuronal regulation of pulpal blood flow is primary controlled by afferent

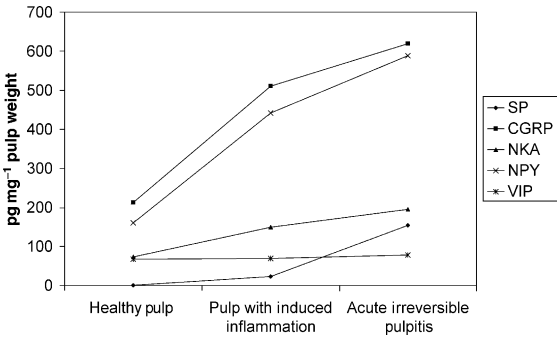

Figure 1 Neuropeptide expression in healthy and inflamed human pulp tissue.

Table 1 Calcitonin gene-related peptide (CGRP) expression in healthy and inflamed human dental pulp

Sample	Healthy pulp	Pulp with induced inflammation	Acute irreversible pulpitis
1	94.33	363.53	545.71
2	180.08	394.38	562.88
3	213.55	494.36	598.49
4	222.04	510.62	619.45
5	253.17	615.83	645.25
6	313.11	685.06	744.70
Mean	212.71	510.63	619.41
SD	73.3	124.0	71.3
ANOVA	$P < 0.0001$*		

Values are given in pg CGRP per mg pulp weight.
*Mann–Whitney's *post hoc* comparisons between groups were also statistically significant.

Table 2 Substance P (SP) expression in healthy and inflamed human dental pulp

Sample	Healthy pulp	Pulp with induced inflammation	Acute irreversible pulpitis
1	0.15	10.84	110.68
2	0.17	13.95	121.20
3	0.28	20.97	124.14
4	0.30	27.50	155.17
5	0.35	29.17	162.78
6	0.72	35.48	252.26
Mean	0.33	22.98	154.37
SD	0.2	9.5	52.1
ANOVA	$P < 0.0001$*		

Values are given in pg SP per mg pulp weight.
*Mann–Whitney's *post hoc* comparisons between groups were also statistically significant.

Table 3 Neurokinin A (NKA) expression in healthy and inflamed human dental pulp

Sample	Healthy pulp	Pulp with induced inflammation	Acute irreversible pulpitis
1	59.04	84.55	153.59
2	61.61	145.30	188.01
3	68.66	149.09	192.87
4	73.35	156.02	195.02
5	83.65	164.08	212.26
6	93.69	195.62	228.15
Mean	73.33	149.11	194.98
SD	13.3	36.4	25.2
ANOVA	$P < 0.0001$*		

Values are given in pg NKA per mg pulp weight.
*Mann–Whitney's *post hoc* comparisons between groups were also statistically significant.

Table 4 Neuropeptide Y (NPY) expression in healthy and inflamed human dental pulp

Sample	Healthy pulp	Pulp with induced inflammation	Acute irreversible pulpitis
1	131.59	305.87	459.21
2	145.49	441.80	551.29
3	157.57	450.23	587.40
4	159.63	466.69	613.70
5	165.79	475.54	620.15
6	202.28	510.74	700.04
Mean	160.39	441.81	588.63
SD	23.8	70.8	80.2
ANOVA	$P < 0.0001$*		

Values are given in pg NPY per mg pulp weight.
*Mann–Whitney's *post hoc* comparisons between groups were also statistically significant.

Table 5 Vasoactive intestinal polypeptide (VIP) expression in healthy and inflamed human dental pulp

Sample	Healthy pulp	Pulp with induced inflammation	Acute irreversible pulpitis
1	73.47	57.17	73.76
2	67.54	83.20	87.23
3	75.06	95.27	98.46
4	54.71	53.14	84.80
5	59.82	64.25	61.00
6	72.10	61.45	62.52
Mean	67.12	69.08	77.96
SD	8.2	16.5	14.8
ANOVA	$P > 0.05$*		

Values are given in pg VIP per mg pulp weight.
*Differences between groups were not statistically significant.

(sensory) and sympathetic fibres. Parasympathetic regulation appears to be insignificant, as VIP levels showed little variation between healthy and inflamed pulps. However, its presence supports the existence of parasympathetic fibres in dental pulp as suggested in previous studies (Uddman *et al.* 1980, Segade *et al.* 1987, Wakisaka *et al.* 1987).

Sensory nerve fibres that contain CGRP, SP and NKA participate in vasodilation and neurogenic inflammation (Olgart 1985). The dental pulp, and especially the subodontoblastic plexus, is highly supplied by such fibres (Byers *et al.* 1987). It is interesting to notice the high basal level of CGRP in healthy pulps, which may be due to its regulating functions in pulp physiology, such as maintaining vascular tone, ensure smooth flow and the consistent supply of nutrients to the tissue,

regulating the interstitial pulpal pressure and stimulating growth of pulpal cells, such as fibroblasts and odontoblast-like cells (Trantor *et al.* 1995, Calland *et al.* 1997, Goodis & Saeki 1997). Previous studies in pulp neuropeptides physiology have shown sprouting of CGRP immunoreactive nerve fibres in response to dentine injury in rat molars and in the presence of inflammation (Taylor *et al.* 1988). Results from the present study correlate with the previous statement as there was a significantly increase in neuropeptide expression in both inflammatory phenomena.

Substance P was the neuropeptide with the lowest expression in healthy pulps and the second lowest in the irreversible pulpitis group. Besides these findings, it is clearly demonstrated that SP plays a major role in neurogenic inflammation, where its increased level may be an effort to regulate pulpal blood flow by controlling the fluid exudate related with the inflammatory phenomenon (Kim 1990, Hargreaves *et al.* 1994, Olgart 1996, Awawdeh *et al.* 2002). An important issue to remember is that SP rarely acts alone, as it coexists together within the same nervous fibres with CGRP and NKA, and it has been hypothesized that SP and NKA have a synergistic effect as they share a common cellular receptor, a 'septide-sensitive' NK1 receptor (Beaujouan *et al.* 2004).

Expression of NKA presented a very similar trend with SP, as both are tachykinins expressed in the same fibres. However, higher expression of NKA could be explained as this neuropeptide may also be expressed in non-neuronal cells such as human endothelial cells and different type of inflammatory and immune cells (Pinto *et al.* 2004).

Neuropeptide Y was the second highest neuropeptide expressed in human pulp. It is highly conserved throughout evolution and is therefore thought to be an important neuropeptide, which modulates numerous physiological processes including blood pressure, appetite and circadian rhythms (Wahlestedt & Reis 1993). High basal levels of NPY may be explained due to this physiological control and as a consequence of numerous sympathetic adrenergic fibres present in dental pulp, predominantly associated with blood vessels within the root and mid-coronal region. It has been demonstrated that variations in adrenergic activity could induce changes in dentinogenesis through blood vascular changes or modulation of odontoblast metabolism (Avery *et al.* 1980, Rodd & Boissonade 2002). Although sympathetic fibres do not reach the odontoblastic layer, during the inflammatory process, NPY expression increases as it plays an important role in sympathetic vasoregulation, enhancing noradrenaline-evoked vasoconstriction at the postsynaptic level as a response of intense vasodilation produced by neuropeptides released from sensory fibres (Kim *et al.* 1996).

Vasoactive intestinal polypeptide was the only neuropeptide which expression remained almost static in the three groups. This finding could be explained as VIP-expressing fibres are often found as free endings in the stroma with no obvious relation to blood vessels (Wakisaka *et al.* 1987). There is evidence of VIP-immunoreactive fibres presence in intact permanent teeth within the pulp horns, which ran parallel to the horn and did not appear to be related to any other structures. However, there is no evidence of any VIP-positive fibres extending into the odontoblast layer, which may be a reason why VIP expression remains unaltered during the inflammatory phenomenon (Rodd & Boissonade 2002).

In general, there was a greater expression of neuropeptides in acute irreversible pulpitis than in pulps having induced inflammation. This finding could be explained by the evolution time of the inflammatory process. In the induced pulpitis group, pulps were obtained 10 min after the stimulus was applied whilst inflammation in pulp tissue having a clinical diagnosis of acute irreversible pulpitis had at least 24-h duration. The higher neuropeptide expression in the acute irreversible pulpitis group could be regulated by two different mechanisms: there might simply be enhanced by the nerves subpopulation that normally express them or there might be *de novo* synthesis of certain peptides by nerve subpopulations that do not normally express them. The experimental method used in this study was unable to establish which mechanism occurred. However, there is good evidence to suggest that both of these mechanisms may be involved during acute irreversible pulpitis (Rodd & Boissonade 2002).

Only non-smoking human donors were included in this study, as it has been proved that the concentration of CGRP is significantly higher in smokers (Awawdeh *et al.* 2002). It has also been demonstrated that nicotine triggers the release of SP, NKA and CGRP from C-fibres in the lungs and increases the expression of NKA and CGRP in the pulmonary effluent (Lee *et al.* 1995).

The local anaesthetic used in this study was 4% prilocaine without vasoconstrictor to prevent neuropeptide expression becoming attenuated by α-adrenergic agonists (e.g. vasoconstrictors) as stated by others (Pertl *et al.* 1997, Hargreaves *et al.* 2003). There was a

10-min delay after exposing the pulp in the induced inflammation group before proceeding with tooth extraction. It has been shown that this period of time appears to be sufficient for allowing the neuropeptide to be released from terminal fibres (Hargreaves *et al.* 2003, Caviedes-Bucheli *et al.* 2004a, 2005a,b). Mechanical pulp exposure was successful in inducing inflammation as stated in previous studies, where it has been demonstrated that high-speed drilling and mechanical pulp exposure are effective stimulus to release neuropeptides in dental pulp (Buck *et al.* 1999, Caviedes-Bucheli *et al.* 2005a).

Neuropeptide expression was measured by RIA, as it has been a technique widely used for determining and quantifying the presence of peptides, such as hormones, growth factors and neuropeptides due to its high sensitivity for measuring low molecular weight peptides in very small concentrations (Caviedes-Bucheli *et al.* 2004b, 2005a,b).

It should be noted that teeth from both control groups were caries and restoration free; it is thus important to be aware of the limitations of the findings. It has been demonstrated that caries-affected teeth show a significant increase in SP, CGRP, VIP and NPY expression with caries progression (Rodd & Boissonade 2002).

Although the present findings cannot be extrapolated to resolve current clinical endodontic problems, it is conceivable that future clinical therapies could be aimed at manipulating neuropeptide expression or blocking their receptors, in order to modulate a variety of biological mechanisms. The use of neuropeptide antagonists should also be investigated, as in migraine, where this type of therapy has shown optimistic results (Dennis *et al.* 1990, Edvinsson *et al.* 1998).

Conclusions

Expression of CGRP, SP and NKA released from C-fibres and NPY released from sympathetic fibres is significantly higher in the inflamed human pulp compared with healthy pulp. Expression of VIP released from parasympathetic fibres is not increased during the inflammatory conditions of human dental pulp.

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