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QUANTITIVE EVALUATION OF DENTIN SIALOPROTEIN (DSP) USING MICROBEADS - A POTENTIAL EARLY MARKER OF ROOT RESORPTION

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SUMMARY
Purpose. This study had the aim of comparing two different methods of analysing dentin sialoprotein (DSP) in the gingival crevicular fluid (GCF): the conventional ELISA approach and a new method involving the use of magnetic micro-beads coated with an antibody specific for DSP prior to ELISA analysis.
Materials and methods. GCF was taken from six patients following twelve weeks of orthodontic treatment using paper strips inserted into the mesial and distal sulci of the upper incisors, and analysed using both methods.
Results. Statistical analysis of the results using the Mann-Whitney non-parametric test showed that the micro-bead approach conferred more reliability and less variability on the conventional ELISA approach. Furthermore, this method, for the first time, enables the quantification of the DSP in the sample in ng/μl.
Conclusions. The innovative micro-bead/ELISA approach proposed provides a reliable means of quantifying the DSP in the GCF.

Key words: sialoprotein, marker, resorption, tooth, root.

Introduction

External apical root resorption (EARR) is a condition that may arise during orthodontic treatment through a complex combination of factors such as individual susceptibility and the application of mechanical forces. EARR can be achieved also in non-orthodontic situations (1-4). This leads to the loss of dentine and cementum from the radicular apex, and may thereby even shorten the tooth root (5). In permanent teeth it is an inflammatory pathological process due to the prolonged action of polynuclear cells, which intercede when the surface of the root is damaged (6). Hence, external root resorption, which is typically localized at the apex, may be provoked by orthodontic treatment. This is to be avoided if possible, as it may adversely affect the vitality of affected teeth and establish an unfavourable relationship between their crown and root, making them unsuitable for use as anchorage for prosthetic restorations (7).
Individual susceptibility is thought to be the main determining factor, and this condition may arise in the absence of orthodontic treatment. However, orthodontic forces, as well as other patient-dependent factors, can increase the risk (8, 9) and lead

to the development of mild, moderate or severe resorption. Mild EARR is generally thought to be of negligible clinical significance, but severe forms, sometimes, may even lead to the loss of the entire apical third of the tooth root (10).

Hence early diagnosis is indispensable for the identification of teeth at risk of severe resorption, but radiography, the only present validated option, reveals EARR when 60-70% of the mineralized tissue has already been lost (10), i.e., 5-6 months into the treatment. This means that the small lesions, which can arise after as little as 7 weeks of therapy, as confirmed histologically, cannot be detected by such methods (11). Moreover, being two-dimensional, these images cannot tell us whether resorption is in the active phase. Despite these considerable drawbacks, X-ray is commonly used for this purpose, because it is easy to use and relatively inexpensive (12). Although histological examination has revealed the presence of orthodontically induced EARR in 90% of teeth (13), this number is considerably reduced when radiography is the sole means of investigation. Although the advent of 3D technology has greatly improved the quality of radiographic images, the issue of invasiveness still remains to be resolved. Indeed, multiple scans are required to diagnose the progression of the disease (14).

This has prompted search for alternative tests, such as immunoassay, based on the identification of dentin sialoprotein (DSP) in the gingival crevicular fluid (GCF). Being a dentine-specific matrix protein involved in the mineralization of predentine into dentine (15), its presence in the crevicular fluid therefore indicates that resorptive processes are in progress (10, 16, 17). The aim of this work was to determine whether an innovative adaptation of the existing ELISA assay, that introduces magnetic micro-beads coated with an antibody specific for DSP prior to ELISA analysis, is more or less reliable than its precursor in detecting this protein marker in the CGF.

Materials and methods

Six patients were subjected to CGF sampling and DSP assay at 12 weeks following the start of orthodontic treatment. The patients, 5 females and 1 male with an average age of 14 years, were all undergoing fixed orthodontic treatment by means of Damon appliances. None presented systemic diseases, poor oral hygiene, poor motivation, caries or pathologies of the pulp, pocket depth of more than 3 mm or bleeding on probing, none had previously undergone orthodontic treatment with fixed appliances, and none had taken antibiotics in the preceding 6 months or anti-inflammatories in the month prior to the study.

GCF collection

The sampling site was gently washed and dried using low-pressure water and air jets, respectively, taking care not to trigger bleeding. Cotton wool rolls and saliva ejector were used to keep the area contamination-free while sterile paper strips (PerioCol Paper Strips, Oraflow®) were inserted to a depth of 1-2 mm into the mesial and distal gingival sulci of the upper central and lateral incisors for 1 minute to withdraw a sample of GCF from each (Figure 1).

Immediately after removal, each paper strip was sealed in a centrifuge tube containing a 1 x phosphate-buffered saline solution and 0.1 mM of the protease inhibitor phenylmethyl sulphonyl fluoride (PMSF). After the initial samples were collected, a second set was taken, following the same procedure, after an interval of 1-2 minutes, in order to have two measurements of the same site and create an average. Both sets of samples were then sent to the laboratory for analysis (Figure 2).

Laboratory analysis

Each collected sample of GCF was analysed using two techniques, the conventional ELISA

Figure 1
The sterile paper strip is inserted into the gingival sulcus to a depth of 1-2 mm and left in place for 1 minute.

method, and the micro-bead adaptation of the same proposed herein. The conventional ELISA detection method consists of two principal steps:

a) Sampling and storage of the GCF.
To recover the GCF, the paper strips were eluted by centrifugal filtration at 15,000 g for 5 minutes. This was performed twice to ensure that as much protein as possible was recovered, as previous studies have shown that only 83-91% is collected by the second elution (18).

b) ELISA analysis.
Non-competitive indirect ELISA was used to determine the presence of the DSP in the GCF samples collected. The primary antibody was used at a dilution of 1:1000 and the secondary antibody at a dilution of 1:2000. Micro-well plates were read by ELISA Anthos 2010, and the OD/µl value obtained indicated the amount of DSP present in each sample.
The same samples were then subjected to the novel micro-bead approach, which was identical to that described above, except for the addition of an intermediate step between the sampling and storage of the GCF and the ELISA analysis. This involved the use of magnetic MagSi Protein A and G micro-beads to selectively capture the DSP. These silica beads (Figure 3) are coated with either protein A or G bonded to the required specific antibody – in this case DSP-goat polyclonal IgG. The advantage of using these beads, with respect to those coated with streptavidin (a protein with a high affinity for biotin) is that no biotinylation is required and the binding is reversible, making them ideal for isolation of proteins and proteomes. Furthermore, the magnetic properties of these beads enable rapid and easy washing to isolate the protein.

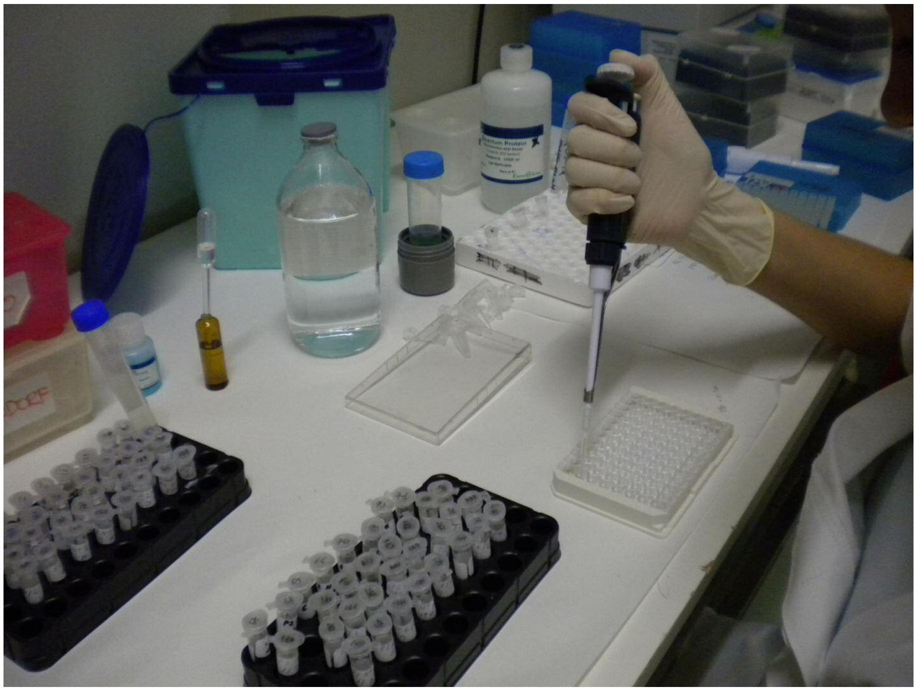

Figure 2
Centrifuge tubes containing the paper GCF-collection strips during the laboratory analysis.

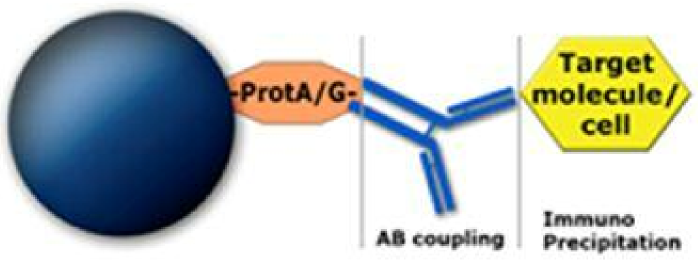

Figure 3
MagSi micro-beads (MagnaMedics®) coated with protein A or G and bound to specific antibodies (i.e., DSP-goat polyclonal IgG), which selectively bind to the target protein (i.e., DSP).

Following the ELISA analysis, performed as above, the laboratory was provided with the purified DSP peptide, which presents the same immune epitope against the antibody, so that it could generate a calibration curve (Figure 4) to define the specific relationship between protein and antibody. This allowed the results of the ELISA test, i.e., the amount of DSP detected, to be quantified. In case of analysis of dentin sialoprotein (DSP) in the gingival crevicular fluid local anesthesia can be performed to sampling patients but it may have relevant side effect (19-22) and severe complications (23). This topic can be also potentially investigated with immunofluorescence techniques which are well known since the nineties (24, 25).

Statistical analysis

This was performed to describe and quantify the data acquired from the samples, and to identify any variability therein. The means and standard deviations provided by each method for each patient were calculated, and the two tests were subjected to the non-parametric Mann-Whitney test for independent comparison.

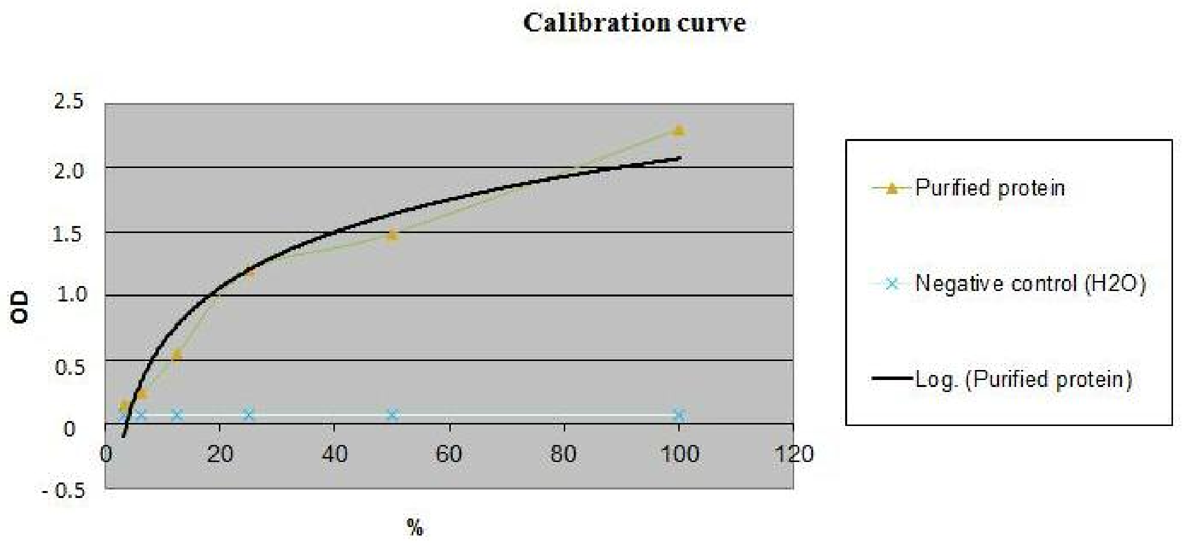

Figure 4
Calibration curve showing the behaviour of the purified protein and antibody.

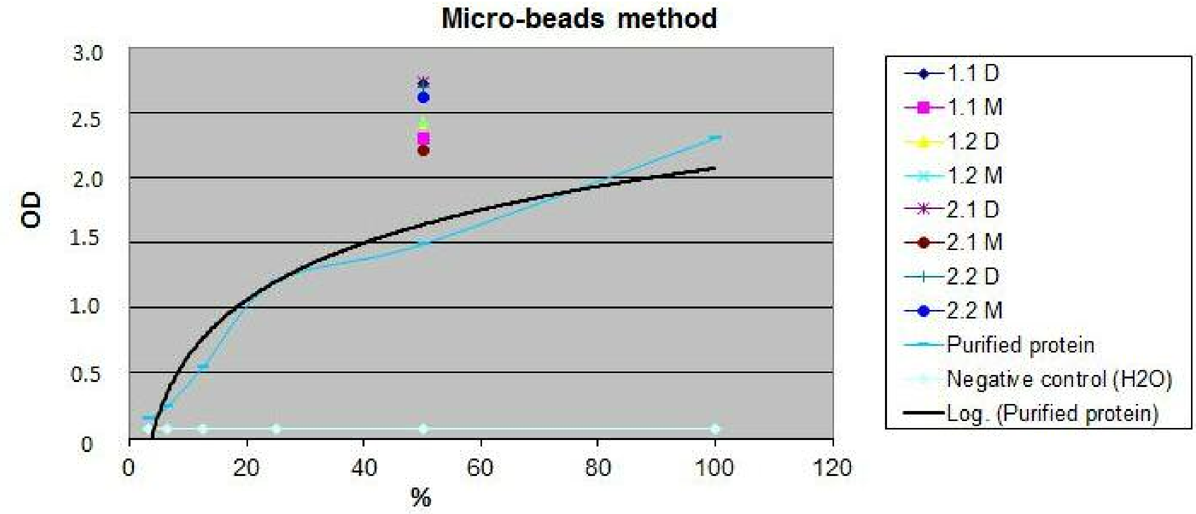

Figure 5
Graph of ELISA test results obtained with the mean of the measurements for the six patients at each site using the micro-beads method.

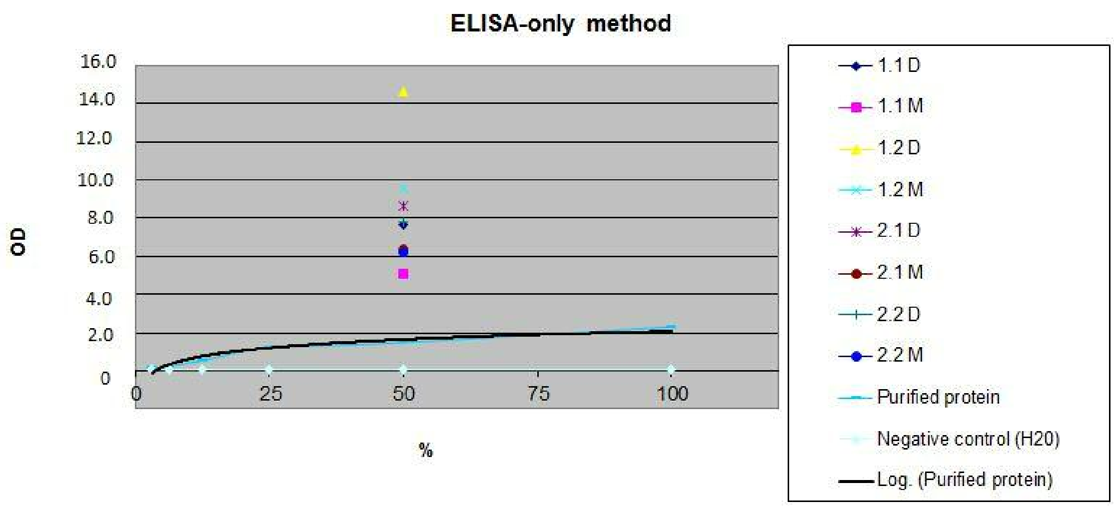

Figure 6
Graph of ELISA test results obtained with the mean of the measurements for the six patients at each site using the ELISA-only method.

Results

The ELISA results were plotted as graphs of the means obtained for each site in the group of six patients. The differences between the two methods are correctly reported in the graphs (Figures 5 and 6) and in the Table 1 that shows the normalized values of OD (DSP)/200µl yielded by ELISA analysis of the samples. These values have been statistically analysed and the results showed the differences in means and standard deviations obtained for each patient by each analytical method (Table 2). The disparity of the results obtained by the two methods is clearly showed in the graph of the non-parametric Mann-Whitney test (Figure

Table 1 - Normalized values of OD (DSP)/200µl detected at each sampling site (as indicated alongside) in each patient (in columns). "D" and "M" are respectively the distal and the mesial gingival sulci of the inticated tooth.

ELISA only method OD (DSP) / 200µl	**Patient 1**	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**	**Patient 6**
1.1 D	5,67	11,75	2,32	10,31	5,00	10,79
1.1 M	4,90	4,64	0,83	6,49	5,38	8,01
1.2 D	6,75	46,94	8,72	8,89	5,97	10,59
1.2 M	5,15	16,79	4,18	7,12	8,15	15,83
2.1 D	7,03	12,25	5,83	8,63	5,34	12,69
2.1 M	5,30	10,41	0,68	5,83	5,99	9,97
2.2 D	6,53	9,57	8,84	7,52	4,27	10,17
2.2 M	5,19	9,06	3,31	6,47	4,45	8,86
Mean	5,81	15,17	4,34	7,66	5,57	10,86
Micro-beads method OD (DSP) / 200µl	**Patient 1**	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**	**Patient 6**
1.1 D	2,88	3,08	2,97	2,16	3,31	1,97
1.1 M	2,41	2,73	2,41	1,93	2,28	2,07
1.2 D	1,95	2,76	2,50	2,60	2,18	2,55
1.2 M	1,66	2,73	2,84	2,51	2,31	2,47
2.1 D	2,08	2,70	3,15	2,97	3,19	2,30
2.1 M	2,29	2,58	2,88	2,17	2,60	0,79
2.2 D	1,60	2,86	3,06	2,74	3,01	2,89
2.2 M	2,55	2,77	3,00	2,78	3,10	1,52
Mean	2,18	2,78	2,85	2,48	2,75	2,07

7). Furthermore the employment of the calibration curve in the micro-beads method has given the possibility to obtain a concentration in ng/µl of DSP (Table 3).

Discussion

External apical root resorption is a common, yet unexpected phenomenon associated with orthodontic tooth movement. Early detection of small root resorptions during orthodontic treatment is essential for identifying teeth at risk of severe resorption.

This study was designed to improve on an existing method for detecting dentin sialoprotein (DSP), a marker for external apical root resorption (EARR) in the gingival crevicular fluid (GCF) of patients undergoing orthodontic treatment. All our samples showed traces of this protein, confirming previous findings by Balducci

Table 2 - Results of the statistical analysis in OD (DSP) / 200μl, indicating mean and standard deviation obtained for each patient with both methods.

	Micro-beads method [values in OD (DSP) / 200μl]		**ELISA only method [values in OD (DSP) / 200μl]**	
	Mean	Standard deviation	Mean	Standard deviation
Patient 1	2,18	0,44	5,81	0,83
Patient 2	2,78	0,15	15,17	13,28
Patient 3	2,85	0,26	4,34	3,22
Patient 4	2,48	0,36	7,66	1,51
Patient 5	2,75	0,46	5,57	1,22
Patient 6	2,07	0,66	10,86	2,44

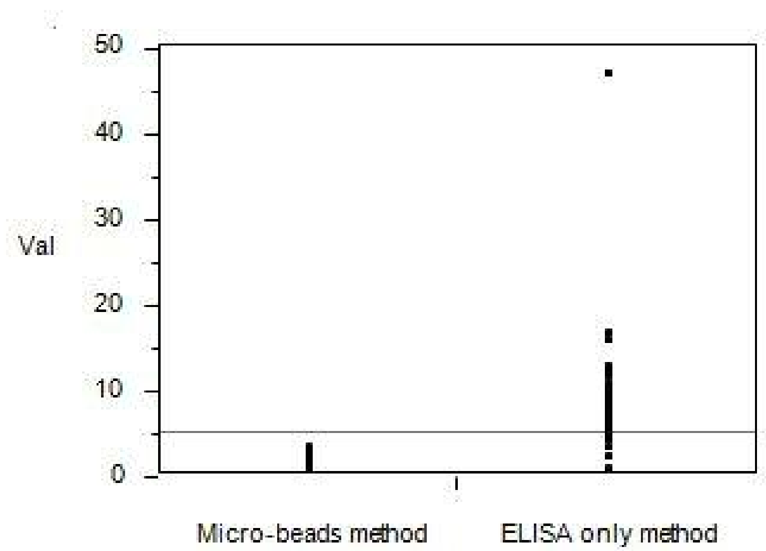

Figure 7
Results of the non-parametric Mann-Whitney test.

(10) and Kereshanan (17). This appears to reflect the complex cellular and structural rearrangements that take place in the root even without exposure to orthodontic forces. Indeed, dentine is not a homogeneous tissue, and its components change with the age of the patient and the degree of maturation of the teeth (26). Odontoblasts and odontoclasts could be working in a similar manner to the osteoblasts and osteoclasts of bone to form, resorb, remodel and maintain dentine (16).
The use of radiography as the sole diagnostic means of distinguishing whether or not root resorption is under way significantly limits research into the matter, as it is unable to provide information on the extension, state of activity or three-dimensional localization of the disease, or indeed whether it is caused by a physiological or pathological processes. Only histological examination is able to provide solid, definitive answers to these questions, but was obviously unsuitable for use in this study.
The study by Qin et al. (27) raised the theory that

Table 3 - Concentration of DSP in ng/µl obtained with the micro-beads method by means of the calibration curve.

ng/ul	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
1.1 D	15,431	21,599	18,108	4,827	31,492	3,502
1.1 M	7,171	12,149	7,253	3,294	5,804	4,164
1.2 D	3,392	12,790	8,333	9,874	4,995	9,056
1.2 M	2,118	12,109	14,527	8,463	6,101	8,019
2.1 D	4,212	11,654	24,056	17,946	25,659	6,061
2.1 M	5,948	9,495	15,545	4,878	9,762	0,511
2.2 D	1,930	14,972	20,940	12,349	19,079	15,788
2.2 M	9,130	13,064	19,033	13,171	22,243	1,679

DSP may not be entirely dentine-specific. Indeed, both DSP and DPP (dentine phosphoprotein), two non-collagenous components of dentine's organic matrix, are expressed by the same messenger RNA (mRNA) transcript, which codes for a protein precursor called DSSP, which was previously considered specific for the dentine. However, Qin (27) discovered, by Western blot, that the DSPP gene is also expressed by osteoblasts, specifically in the long bones of cats, in whose extracts it was detected at roughly 1:400 that found in dentine. Furthermore, by means of inverse polymerase and specific primers in the 5' (DSP) and 3' (DPP) portion, DSPP mRNA was found in osteoblast-like cells and murine cranial osteoblasts, and this gene is expressed in far lower levels in osteoblasts with respect to odontoblasts. Although expression of the DSPP gene in the bone is low, it may account for the slight traces of DSP detected in the GCF of all patients.

A final note on this subject, although not yet demonstrated in the literature, Kereshanan, that has shown an increase in DSP values in all samples at 12 weeks following the start of orthodontic treatment (17), postulates that the cementum too contains DSP in its matrix. If confirmed, this could indicate that DSP release into the GCF is the natural consequence of physiological remodelling if the tooth root, in particular the cementum, during orthodontic treatment.

The non-specificity of DSP was also investigated by Baba (28), who studied the formation of immature rat first molars using immunohistochemical and *in situ* hybridization techniques, the former by means of specific anti-DSP polyclonal and monoclonal antibodies, and the latter by means of RNA probes to detect the DSP transcripts. The results of this investigation suggest that in the initial stages of embryogenesis of the periodontium, DSP is also synthesized and secreted by osteocytes, cementoblasts, cementocytes and fibroblasts, but it was not detected in the acellular cementum. Based on the *in situ* hybridization findings, the Author hypothesized that DSP expression in the alveolar bone, cellular cementum and periodontal ligament is transitory, given that it is only detected, at low levels, in a limited time window corresponding to the formation of these tissues.

In an article by Burgener (29), starting with the idea that low levels of DSP are expressed in the bone (27), the hypothesis that teeth diagnosed with periapical periodontitis feature higher levels of DSP in the GCF than healthy teeth was explored, and showed that this was not in fact the case and that DSP is therefore not a suitable marker for diagnosing apical periodontitis.

Although no trauma patients were included in our study, it is interesting to note that Kumar (30) investigated the amount of DSP in the GCF of teeth with trauma-induced root resorption. The results showed that it is possible to measure a considerable quantity of DSP at all such teeth two weeks after the traumatic event, without this being measurable on radiographic examination.

The search for markers in the gingival crevicular fluid is a safe, non-invasive, site-specific method of early diagnosis of active root resorption, as stated by Mah and Prasad (16). Our study is the first to attempt to evaluate this marker using DSP-specific micro-beads, and therefore the values we obtained in ng/µl cannot be compared with any literature to date. We hope, however, that due to its advantages this approach will be subject to further study so that such a comparison may be made in the future.

The homogeneity of the results obtained by means of the micro-bead approach was confirmed by the statistical analysis of the data pertaining to the description of each patient, in particular mean and standard deviation (Table 2). Furthermore, a statistically significant difference between the two methods is evident (Figure 7). In fact all the values obtained with the micro-bead method are more uniform than those obtained by the traditional method. This is presumably attributable to the specificity of the antibody coating on the magnetic beads (i.e., DSP-goat polyclonal IgG), which prevents cross-reactivity between the antibody and other components of the GCF with similar chemical structures to DSP. In addition to improved precision of ELISA readings, this technique, through the introduction of purified DSP, also enables the trend in the reaction between the antibody and the increase in the protein upon ELISA testing to be described (Figure 4), as well as the quantification of the protein itself in the gingival crevicular fluid withdrawn in µg/ml for each patient at each sampling site (Table 3).

This is indisputably a boon when compared with the ELISA-only approach, which by its very nature furnishes partial, indirect and relative results, and which is unable to provide an absolute value for the marker (i.e., in µg/ml). The micro-bead refinement, on the other hand, by enabling a calibration curve to be plotted, can be used to quantify the amount of the protein marker in the GCF. This overcomes the difficulty found in the past in definitively quantifying the protein, when identification of DSP was determined not on the basis of an absolute numerical value but on a relative evaluation expressed as a percentage of the total protein content of the GCF. The potential risk is that any variation in the global protein content of the GCF during treatment can have an indirect effect on the DSP concentration measured by ELISA.

We are nevertheless aware that, to perform an absolute quantification of the protein in the gingival crevicular fluid, quantification of the fluid taken in the sample is essential. This would guarantee the accuracy of the quantitative value expressed at the end of the test. The use of a tool that can reveal the quantity of fluid in the sample (e.g., Periotron by Oraflow®) would enable the calculation of the absolute concentration of the protein in a known quantity of fluid, which would exclude the inevitable differences in sample volume inherent in the sampling procedure itself.

Conclusions

The results obtained indicate that the modified micro-bead approach employed herein is a more reliable means of assessing the GCF proteins than the traditional ELISA-only method. This is confirmed by statistical analysis, which demonstrated a regular trend in the data obtained using the micro-bead technique, as compared to that acquired by means of the conventional approach, whose data was less evenly distributed.

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