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Abstract

Advanced Glycation End Products in the Dental Pulp

Brynne Ann Reece, Master of Science, 2015

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Hyperglycemia can increase peripheral advanced glycation end products (AGE), with associated diabetic neuropathic complications. An association between glycemia and pulpal AGE, has not been elucidated, nor has the feasibility to measure pulpal AGE. The primary aim was method development to measure pulpal AGE. Secondary aims were to determine links between glycemia and local AGEs, via comparisons between HbA1c, pulpal AGEs (in inflamed vs. non-inflamed pulps), and electronic pulp test (EPT) results. Twenty-three patients underwent venipuncture and vital pulp sampling during endodontic treatment. Local Nε- carboxymethyl(lysine) (CML), peripheral CML and glycosylated hemoglobin (HbA1c) were compared using enzyme-linked immunosorbent assay (ELISA). CML values were normalized to protein via bicinchoninic acid (BCA) assay. Pearson's correlation was used to evaluate relationships between HbA1c and pulpal AGE, plasma AGE, and EPT readings. One-way ANOVA was used to determine differences between pulpal AGE in inflamed and non-inflamed pulps. **Results:** A significant correlation was observed between pulpal and plasma AGE (r=.50, p=.025). There was a significant inverse relationship between pulpal AGE and EPT values (r=-.41, p=.036) and a non-significant inverse relationship between plasma AGE and EPT (r=-.25,

p=.16). There were no significant correlations between pulpal AGE and HbA1c (r=-0.08, p=0.37), or plasma AGE and HbA1c (r=0.14, p=0.29); no differences in pulpal AGE between inflamed and non-inflamed pulps (F=1.64, p=0.22). **Conclusions:** A laboratory method was developed by which pulpal AGE could be measured and used for analysis of biomarkers. There was a significant correlation between pulpal and plasma AGE.

Advanced Glycation End Products in the Dental Pulp

By Brynne Ann Reece

Thesis submitted to the Faculty of the Graduate School

of the University of Maryland, Baltimore in partial fulfillment

of the requirements for the degree of

Master of Science

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To my family, with love.

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REVIEW OF THE LITERATURE

Introduction

Diabetes mellitus represents a major healthcare problem, associated with significant morbidity and mortality, as well as steadily increasing economic costs. In fact, the most recent international assessments from the World Health Organization (WHO) and the International Diabetes Federation (IDF) names diabetes mellitus among the top ten leading causes of death, affecting approximately 387 million people worldwide (1, 2). According to the Centers for Disease Control and Prevention, the most recent national healthcare costs for diabetes alone approximates $245 billion (3), thus clearly posing substantial health and economic challenges. Furthermore, national statistics estimate that 29.1 million Americans have diabetes, and 79 million additional Americans aged 20 and older have pre-diabetes, making them susceptible to developing type 2 diabetes in the next five to ten years.

As a group of complex metabolic disorders, diabetes mellitus is characterized by pancreatic β-cell dysfunction, resulting in little or no insulin production (type I diabetes); insufficient insulin secretion or impaired insulin action on tissue, due to cellular insulin resistance (type II diabetes). Both conditions would result in elevated blood glucose levels. Ultimately, this condition has the potential to alter several immune functions and is associated with chronic inflammation, progressive periodontal destruction, and reduced tissue repair capacity (4). Hyperglycemia appears to be the main cause of diabetic complications, with oxidative stress playing a major role in hyperglycemia-induced tissue injury (5). Furthermore, biomarkers known as advanced glycation end products (AGEs)

have been investigated for their pathologic involvement, as their accumulation appears to be linked to hyperglycemic environments. These compounds represent a group of modified proteins or lipids with inherent tissue damaging potential (Fig.1). Thus, they have been implicated in the genesis of diabetes-induced micro- and macrovascular complications, as well as diabetes-associated periodontal disease. AGEs' direct and indirect pathologic effects include interactions between AGEs and the receptors for AGE (RAGE), as well as oxidative-stress-mediated pathways, respectively. The findings of this study, with emphasis on AGEs' indirect pathologic effects may elucidate associations between hyperglycemia, AGE, and pulpal neuropathy. Furthermore, it may explain how such a prevalent systemic condition like diabetes may have an effect on local tissue changes in the dental pulp.

The prevalence of diabetes mellitus, as well as its societal and biological implications underscore not only the need for increased screening and diagnostic efforts, but also serious consideration for its effect on oral inflammatory processes. The role of AGEs, in particular, may provide important information regarding their contribution to the pathophysiology of vascular disease, neuropathy, and the reduced tissue healing capacity observed in patients with diabetes. The glycosylated hemoglobin (HbA1c) test represents the standard of care for the diagnosis of diabetes, and for quantifying long-term glycemic levels in patients with diabetes. Links between HbA1c and plasma or serum AGE have been evaluated, but the association remains somewhat unclear, particularly with respect to oral inflammation and local tissue healing potential. Increased knowledge of mechanistic links between hyperglycemia, AGE and oral disease may provide an

additional clinically relevant biomarker which may be useful in the screening or assessment of diabetes mellitus.

Diabetes and Oral Health

Diabetes has been studied in the context of oral health and its potential to exist as a systemic risk factor for the development and progression of severe periodontitis as well as its inverse potential to become poorly controlled as a result of advanced periodontal disease (6, 7). Diabetes and periodontal disease both represent highly prevalent, chronic inflammatory conditions, of which scientific evidence supports an independent bi-directional association (6). Although it has been established that uncontrolled diabetes serves as a risk factor for periodontal disease, the effect of periodontal disease or periodontal treatment on diabetes outcomes remains somewhat unclear. Evidence suggests that oral microbial entrance into the circulation, due to periodontal disease, may result in systemic inflammation, as evidenced by elevated serum levels of acute-phase reactants and biomarkers of oxidative stress (5). If this environment is maintained, as in untreated periodontal disease, this may conceivably contribute to β-cell dysfunction, insulin resistance, and the development of type 2 diabetes (5). Furthermore, considering the fact that the onset of diabetes is preceded by inflammation, it is logical to presume that comorbidities (such as periodontal disease) with the potential to contribute to systemic inflammation may likely increase the risk of developing or worsening diabetes.

Periodontal disease is characterized by dysregulation of infection-related immune responses, which are responsible for host tissue destruction, and eventual tooth loss (7).

Many studies have investigated the link between diabetes and periodontal disease. In 1996, Taylor et al evaluated data from the longitudinal study of residents of the Gila River Indian Community and demonstrated an association between severe periodontitis and increased risk of poor glycemic control (8). In 1998, Grossi and Genco elucidated the two-way relationship between diabetes and periodontal diseases, where they demonstrated, not only that diabetes represents a serious risk factor for severe periodontitis, but that advanced periodontal disease can complicate metabolic control and increase the severity of diabetes (6). It has been further demonstrated that elimination of periodontal infection via systemic antibiotics can reduce glycated hemoglobin levels (9) and insulin requirements, thus improving diabetic status. These findings ultimately reveal a dual pathway of tissue destruction, indicating the importance of controlling chronic periodontal infection as an essential factor for achieving long-term control of diabetes mellitus. Chapple and Genco further emphasized this bidirectional relationship between diabetes and periodontitis via an epidemiological summary of cross-sectional, prospective, and interventional studies. The authors concluded that patients with diabetes maintain an increased risk for periodontal disease. In addition, glycemic control may be more difficult for patients suffering from periodontal disease, as they appear to be at a higher risk for developing diabetic complications, such as cardiovascular and kidney diseases (9). In a meta-analysis with five included studies, it was shown that periodontal treatment (scaling and root planning with or without systemic administration of antibiotics) in patients with type 2 diabetes can improve HbA1c values by 0.40% after three months (10). However, a multicenter, randomized, single-masked, clinical trial with a 6-month follow up period reported no significant change in HbA1c between

baseline and the 3 or 6-month visits in the treatment or control groups. Furthermore, the target 6-month HbA1c reduction of 0.6% was not achieved. Therefore, it is unclear whether non-surgical periodontal treatment for patients with diabetes mellitus is effective in reducing HbA1c (11).

Research also exists to establish the association between diabetes and apical periodontitis (AP). AP is a characterized as a chronic inflammatory lesion located around the apex of teeth with infected root canals. Similar biological pathways appear to link chronic periodontal disease, and chronic endodontic disease to systemic disorders (12). Kakehasi et al established a bacterial etiology for pulpal necrosis and subsequent AP in the 1960s (13). Since that time, similar microbiological characteristics have been shown to exist in periodontal and endodontic diseases (12, 14). Both types of infections are associated with complex microbial profiles, with a predominance of anaerobic gram-negative rods. Both diseases also share a pathologic inflammatory component, characterized by bone resorption and increased local levels of inflammatory mediators and antibodies, including but not limited to TNF-α, IL-1β, PGE2, IL-8, IgA, IgG (15).

Several authors have thus investigated the association between AP and diabetes. Fouad et al (16) induced periapical lesions in first molars of non-obese diabetic mice and found a much more severe inflammatory reaction in the diabetic animals as compared to the control group. Iwama et al (17) had similar findings when evaluating the effect of type 2 diabetes on the inflammatory response, in diabetic rats, after intentional pulp exposure of mandibular molars. They found much larger periradicular lesions after four weeks as

compared to the control group. Garber et al (18) showed reduced pulpal healing by lack of dentin bridge formation and increased inflammatory cell infiltration due to hyperglycemia in diabetic rats. To date, human studies have shown that diabetes mellitus may be associated with a higher prevalence of apical periodontitis; a greater likelihood of asymptomatic periapical infections; delayed or arrested periapical healing; larger periapical radiolucencies and a higher rate of root canal treatment failure (19). In the 1960s, authors investigated endodontic healing in diabetic patients, and demonstrated that patients with poorly controlled diabetes mellitus do not respond to endodontic treatment as favorably as their healthy counterparts(20). Fouad and Burleson (21) evaluated endodontic diagnostic and treatment outcome data in patients with and without Diabetes. They found that diabetic patients had increased periodontal involvement in root filled teeth ($x^2_8 = 20.85$, $p = .008$), and exhibited a lesser likelihood of endodontic treatment success in cases with preoperative periapical lesions as compared to non-diabetic patients ($x^2_1 = 7.2$, $p= .0073$)(21). Most recently, through funding from the American Association of Endodontists (AAE) Foundation, our group (22) demonstrated a positive correlation between glycemic control, as measured by HbA1c and endodontic healing, reflected by lesion size (measured via CBCT) ($r=0.375$, $p=0.027$). One key finding in that study was that several of the self-reported non-diabetic patients had HbA1c values that placed patients in the prediabetes and diabetes range, thus indicating that they were not aware of their diabetic status (22). Their findings highlight the importance of glycemic control as a function of healing potential for endodontic lesions.

Mechanisms underlying Diabetes and Oral Inflammation

Several proposed mechanisms link hyperglycemia to oral inflammation. One early theory maintained that hyperglycemia provided a periodontal environment in which certain bacterial species thrive. Lalla et al investigated this theory by evaluating the effect of hyperglycemia on the subgingival microbial profile. They showed that local hyperglycemia (measured in the gingival crevicular fluid (GCF) of diabetics) might favor the subgingival growth of certain bacterial species, leading to an increased susceptibility to periodontitis and an accelerated disease progression (23). Despite these findings, overall compelling scientific evidence is insufficient to support this theory, since existing studies have not examined the entire periodontal microbiome (9). In the endodontic literature, it has been proposed that patients with diabetes mellitus may be predisposed to more aggressive, symptomatic forms of endodontic infections (21, 24).

It has been suggested that the necrotic pulp environment of diabetic patients may favor a more virulent microbial profile. This theory has fueled investigations into the associations between diabetes mellitus and the presence of specific endodontic microorganisms. In fact, Fouad et al reported a nonsignificant association between type 1 and type 2 diabetes mellitus, and the presence of *P. endodontalis* and *P. gingivalis* in necrotic root canals (24). Diabetes has also been significantly associated with the presence of *Eubacterium infirmum*. Another mechanism underlying the link between diabetes and oral inflammation holds that patients with diabetes may possess altered immunupotency. Salvi et al evaluated links between diabetes and immune cell function, concluding in their study, that monocytes from patients with type I diabetes have a hyper-inflammatory phenotype. Thus, the cells in these patients respond to lipopolysaccharide (LPS) from

periodontal bacteria to produce significantly higher levels of IL-1β, TNF- α, and prostaglandin, than cells from patients without diabetes (25). This altered immune cell function may ultimately result in a reduced healing potential. Finally, numerous studies have explored the role of hyperglycemia and its effect on AGE, which can lead to cellular stress via direct or indirect pro-inflammatory or pro-oxidant cellular influence. Indirect effects of AGE involve its interaction with the receptor for AGE (RAGE), which ultimately leads to inflammation and reduced tissue repair capcity. Direct effects of AGEs include the increased production of reactive oxygen species (6), which in turn stimulate proinflammatory cytokine production through the activation of intracellular pathways (26).

Advanced Glycation End Products

AGEs represent complex compounds that are formed through the non-enzymatic Maillard reaction between reducing sugars or reactive carbonyls with free amino acids (5). Although spontaneously produced in human tissues as a part of normal metabolism and aging, their formation is accelerated in conditions of hyperglycemia and enhanced oxidative stress, thus fueling its implication in the progression of diabetes (27). AGEs are formed through a series of chemical reactions, resulting in intermediate products. These reactions include the formation a Schiff base (as a result of combined elements of glucose and protein molecules, specifically the double bond between the carbon atom of the glucose and the nitrogen atom of the lysine); the formation of an Amadori product (a ketone formed when the hydrogen atom from the adjacent hydroxyl ion combines with the nitrogen atom), and finally the irreversible formation of the advanced glycation end

product (the oxidized Amadori product) (5). Figure 1 illustrates the Maillard reaction, by which the irreversible end product, AGE is produced.

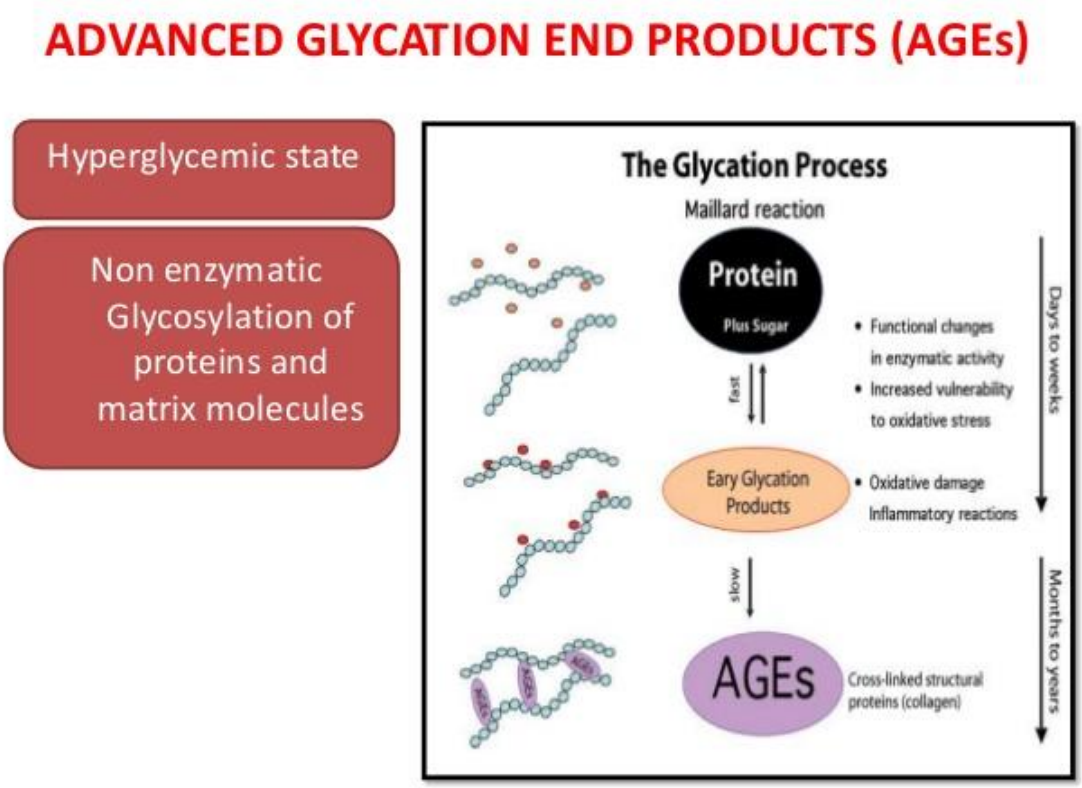

Figure 1: Maillard Reaction and subsequent production of the irreversible product, AGE (28)

Several mechanisms have been proposed to explain how AGE accumulation can cause microvascular damage. First, a direct alteration of protein function in microvascular endothelial cells and neurons, which results in impaired matrix-matrix, matrix-cell, and cell-cell interactions; secondly, alteration in gene expression for molecules involved in vascular and neural pathology by generation of reactive oxidative species (6) and activation of pleiotropic transcription factor, NFκB. Finally, an indirect effect has been proposed via the interaction between the AGE, and its receptor, RAGE, which results in

the perturbation of cellular properties, with a link to sustained inflammation, impaired wound healing and accelerated periodontal disease (29).

Schmidt et al was the first group to demonstrate the expression of AGE in the gingival tissues of diabetic patients with periodontitis (30). Subsequently, other authors have found links between the extent of periodontitis in patients with type II diabetes, and plaque, salivary, and serum levels of AGE (31, 32). In an animal model, Lalla et al demonstrated that *P. gingivalis*-induced alveolar bone loss was increased in diabetic mice compared to non-diabetic controls, and that this bone loss was accompanied by enhanced expression of RAGE, inflammatory AGE, and tissue MMPs in the gingival tissues (33). Thus, it has been definitively shown that a hyperglycemic environment can increase susceptibility to oral inflammation as well as to exacerbate oral disease. Lalla et al (34) identified this central cell-surface receptor for AGEs, termed RAGE (receptor for AGE). This multiligand cell-surface molecule is expressed on various cell types, including endothelial cells, monocytes, smooth muscle cells, and fibroblasts. Studies have shown that the AGE-RAGE interaction plays an important role in the development and progression of diabetic vascular complications (27). In this study, the authors tested the hypothesis that this interaction of critical cells in the diabetic periodontium would contribute to excessive alveolar resorption. Results demonstrated significantly increased alveolar bone loss after *P. gingivalis* inoculation of diabetic mice as compared to non-diabetic mice, indicating a critical link between periodontal infection, exaggerated inflammatory host responses and alveolar bone resorption in a diabetic mouse model (34). In 2010, Pollreisz et al (35) also demonstrated the role of the AGE-RAGE axis in

the amplification of pro-atherogenic responses triggered by *P. gingivalis* in murine aortic endothelial cells (MAEC). The collective data present strong evidence for an indirect association between hyperglycemia, AGE and tissue destruction through the AGE-RAGE complex. It appears that a positive feedback loop results from the AGE-RAGE interaction. This binding activates NADPH oxidases, thus increasing intracellular ROS, which in turn, leads to AGE formation, ultimately triggering AGE-mediated tissue damaging mechanisms. Furthermore, the AGE-RAGE interaction also activates NFκB, which increases the expression of proinflammatory cytokines, such as interleukin 6 (IL-6), monocyte chemoattractant peptide 1 (MCP-1), tumor necrosis factor alpha (TNF-α), vascular cell adhesion molecule (VCAM1), as well as RAGE itself, resulting in an exaggerated inflammatory response (5) (Fig. 2).

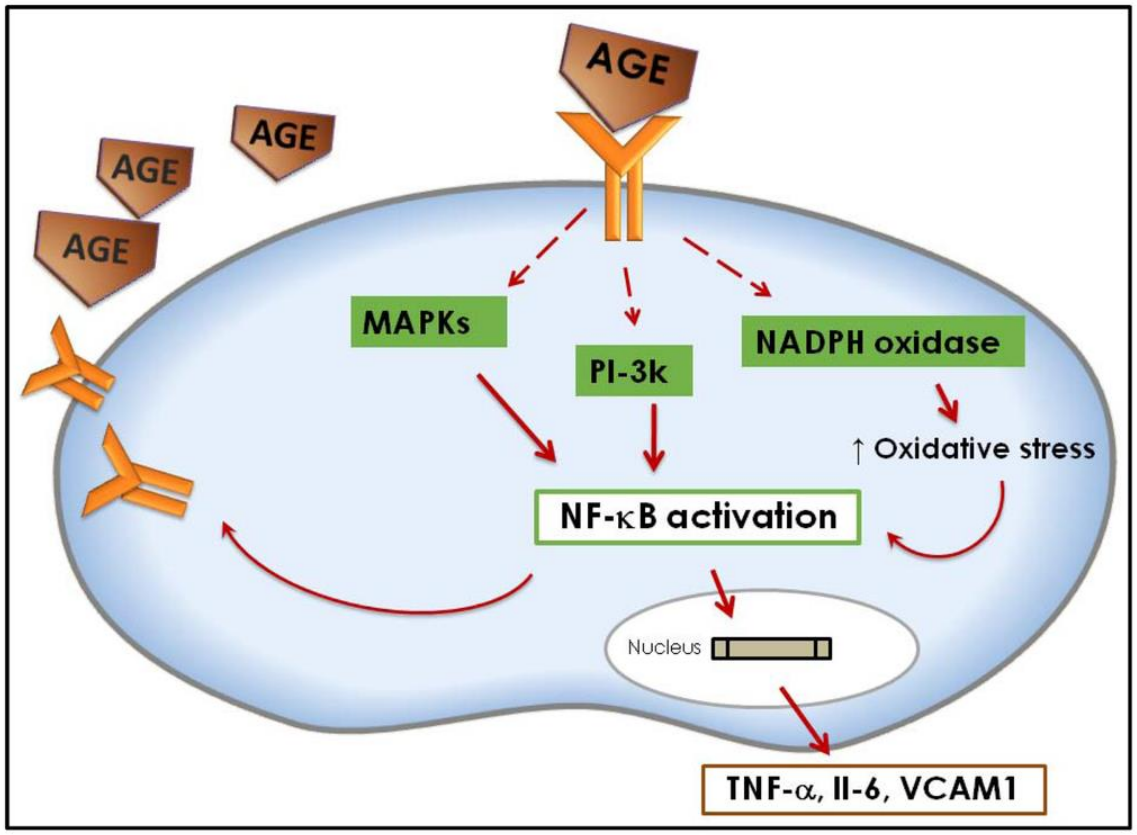

Figure 2: Illustration of AGE-RAGE interaction, resulting in NF-κB activation, increased cytokine production, and ultimate tissue damage (36)

The inherent damaging potential of the AGE results from direct modification of protein structure and function via cross-linking in the extracellular matrix. Modified matrix proteins impair matrix-matrix, matrix-cell, and cell-cell interactions, subsequently causing cell death, cell differentiation, or reduced cell adhesion and migration (5). The cytotoxic potential of AGEs on pancreatic β-cells has been investigated and shown to cause apoptosis of the β-cells, and to stimulate ROS production and induce RAGE expression (37). Thus, AGEs have been shown to represent independent risk factors for the development of insulin resistance through their direct (protein glycation) and indirect (AGE-RAGE) cytotoxic mechanisms (38, 39). Tan et al demonstrated that AGE levels were correlated with insulin resistance in healthy patients via the homeostatic model assessment index (HOMA-IR) (40). Tahara et al also demonstrated that serum AGE levels were independently correlated with the HOMA-IR (n=300) (41).

Hyperglycemia, AGEs, Inflammation and Neuropathy

HbA1c remains the best screening tool for diabetes, as well as the most accurate measure of long-term blood glucose control. However, as an important factor in the pathogenesis of diabetic complications, advanced glycation end-products (AGEs) are also a necessary measure to determine the effect of diabetes at a tissue level, and to assess the presence and/or severity of diabetic complications (5).

HbA1c and AGE are both glycosylated proteins that are increased in the presence of hyperglycemia. The difference between the two compounds lies in the regulation of the glycosylation process, or lack thereof. HbA1c is an early, reversible glycation product which is produced because of a highly regulated, enzyme-mediated process. HbA1c is not inherently pathologic, but is routinely used to screen and monitor pathology (hyperglycemia) because of its direct relationship to blood glucose levels (5). Contrastingly, AGEs are the irreversible end products spontaneously produced via non-enzymatic glycosylation, in the presence of hyperglycemia. AGEs have an inherent, pro-inflammatory tissue damaging potential and contribute to the link between hyperglycemia and diabetic neuropathies. Investigating associations between hyperglycemia, and pulpal AGEs is important in elucidating the effect of diabetes on pulpal inflammation. The findings of this research would benefit diabetic patients by providing knowledge of the relative risk for needing endodontic therapy. If hyperglycemia causes inflammation, and inflammation in turn promotes AGE formation, and AGEs damage tissue, it stands to reason that hyperglycemia may have a deleterious effect on pulp recovery in a case of reversible pulpitis.

Associations between hyperglycemia and neuropathy have been reported in the medical literature, with increased serum accumulation of AGEs being shown to precede, and to accompany histological evidence of diabetic microvascular damage in the retina, kidneys and peripheral nerves (42). In 1993, Bangstad et al reported that diabetic patients exhibited significant increases in glomerulopathy parameters compared to the control group. The observed increased factors included basement membrane thickness,

mesangial volume fraction, matrix start volume and thickeness, insterstitial volume fraction, and mean capillary diameter (43). McCance et al investigated the association between Maillard reaction products and fluorescence in skin collagen from patients with type 1 diabetes. The authors demonstrated significant, independent associations between an AGE precursor Amadori product, fructoselysine (FL), an AGE compound, N^{ε} (lysine) (CML) (Fig.2), and diabetic retinopathy and early nephropathy (44). Their findings suggest a mechanistic link between AGEs and microvascular diabetic complications. In 1997, Berg et al aimed to determine whether circulating AGEs may predict the progression of morphological pathology in diabetic patients with nephropathy. In this prospective clinical trial, kidney biopsies were taken at baseline, and after 24 to 36 months. Each biopsy was analyzed for structural glomerular changes. Retrospective analysis of serum AGEs was correlated to biopsy findings. A stepwise regression analysis revealed that baseline serum AGE levels represented a significant independent variable, whereas the baseline mean HbA1c was not significant in this manner. These findings indicate the predictive value of serum AGEs in the progression of early morphological diabetic nephropathy (45). Therefore, these data demonstrate that AGEs are associated with diabetic complications, including retinopathy and nephropathy. Their involvement makes them a prime target for research into the pathophysiology of diabetes-associated complications.

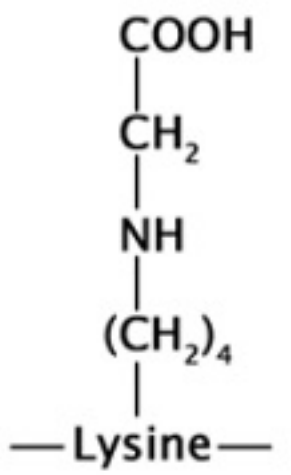

Figure 3: Structure of N^{ε} –(carboxymethyl) lysine (CML) (46)

Electrical pulp testing (EPT) has been used in dentistry from the 1960s. The functionality of this diagnostic tool is based on a threshold stimulus - a measure of the amount of provocation needed to elicit the lowest perceptible intensity of pain (47). Decades before its routine use, associations between EPT readings and histological appearances of pulp tissue were investigated, with inconsistent findings. Several studies reported variable "pain threshold" or electrical stimulus readings within the same pulp status category, begging the question of its reliability for predicting the histological integrity of the pulp. Seltzer et al introduced the wide range of histologic variations associated with pulpal inflammation and infection, indicating the complexity of diagnostic testing and its accuracy in predicting true pulpal status (48). To date, EPT has not been established as a reliable predictor of the degree of pulpal inflammation (49). Nevertheless, within the limited scope of endodontic diagnostic tools, EPT was a viable option with remote potential to quantitatively assess pulpal neuropathy, and the authors sought to investigate its association with pulpal AGEs. Another option, which may be considered for future investigation, is the cold test coupled with patient response to the

visual analog scale (VAS). It has been shown that vital teeth with pain may have more severe histologic conditions (48), which may potentially alter pulpal AGEs. Further research is needed to evaluate the correlation between pulpal AGEs in symptomatic vs. asymptomatic irreversible pulpitis cases (Table 5).

Hyperglycemia and pulpal inflammation

Systemic conditions can reduce the body's wound healing ability. Diabetes mellitus is characterized by hyperglycemia, which inhibits macrophage functions, including chemotaxis and phagocytosis. Hyperglycemia can also cause decreased leukocyte adherence, and cytokine production, as well as increased adherence of microorganisms to diabetic cells (50, 51). The subsequent inflammatory state results in an unfavorable environment for angiogenesis, and cellular proliferation, which constitute critical functions of healthy tissue. Chronic hyperglycemia, in particular, results in protein glycation, which may contribute to the increased susceptibility of these patients to various tissue impairments, such as peripheral neuropathy, vascular insufficiency, and chronic periodontal disease (52, 53).

Garber et al evaluated the potential for hyperglycemia to interfere with oral tissue repair by investigating pulpal healing in rats, subsequent to exposed pulps capped with MTA. Their results demonstrated impaired wound healing in streptozotocin-induced rats compared to controls. Ninety-two percent (92%) of nondiabetic animals showed complete dentin bridge formation compared to fifty-three percent (53%) of diabetic rats. Forty-seven percent (47%) of diabetic rats showed pulpal inflammation, with a

significant inverse association observed between dentin bridge formation and inflammatory cell infiltration (18). These findings indicate the deleterious effect of hyperglycemia on local tissue repair. Therefore, in view of the potential biomarker role of AGE in diabetes-associated tissue injury, we embarked on the present study to investigate the feasibility of AGE measurement in pulpal tissue and the possible validity of this biomarker in diabetes screening and evaluation.

PURPOSE

The primary aim of this prospective clinical study was as follows:

(i) To develop a method to measure AGE in the dental pulp.

Following method development, the secondary aims were described based on sample size having at least 80% power. They are as follows:

(ii) To determine whether advanced glycation end products (AGEs) in dental pulp may be associated with hyperglycemia (45). The association between plasma AGE and HbA1c served as a control.

(iii) To determine if there are differences in the levels of pulpal AGEs between normal and inflamed pulp tissue.

(iv) To determine if there is a relationship between level of pulpal response to electric pulp testing (EPT) and pulpal AGEs.

HYPOTHESES

The primary aim was (i) to develop a consistent methodology by which to measure pulpal AGE. This aim was not measurable and therefore not analyzed statistically. Thus, the three hypotheses, as related to aims ii-iv, analyzed statistically were as follows:

Hypothesis (ii): There is a significant positive correlation between

Hypothesis iia: pulpal AGE and HbA1c

Hypothesis iib: plasma AGE and HbA1c

Hypothesis (iii): There is a significant difference in the level of AGEs between inflamed pulps and non-inflamed pulps.

Hypothesis (iv): There is no significant correlation between

Hypothesis iva: pulpal AGE and EPT values

Hypothesis ivb: plasma AGE and EPT values

MATERIALS AND METHODS

Research Design

This study was conducted in the Advanced General Dentistry, Predoctoral and Postgraduate Endodontic clinics at the School of Dentistry, University of Maryland. Twenty-three patients were recruited from the standard referral pool based on the pulpal diagnosis of normal pulp (elective treatment), reversible pulpitis (elective treatment), and irreversible pulpitis (therapeutic treatment). Six subjects were male and seventeen were female. Informed consent was reviewed and obtained with each participant. All clinical and laboratory procedures were performed in accordance with the protocols approved by the Institutional Review Board of the University of Maryland. Clinical samples were obtained during endodontic treatment, as described in the research protocol, and statistical analysis included an examination of the correlation between HbA1c and pulpal AGE (Table 3); differences in pulpal AGE levels in inflamed and non-inflamed pulps (Table 4); and correlation between pulpal AGE and clinical values (Table 6). A check for $50.00 was mailed to each patient for participating in the study.

The research protocol was approved by the Institutional Review Board of the University of Maryland. Patient recruitment began in August 2014.

Sample Size Determination

Data from Takeda et al (32) was used to perform a power analysis to determine the optimal number of patients for the research study. With a total N of 55, a population correlation of 0.32 against a null = 0, a 1-tailed test, and p=0.05, power was equal to .80.

Inclusion criteria

Patients had to meet the following inclusion criteria to be eligible to participate in the research study:

- Patients had to be age 18 years or older
- Patients were either self-reported diabetics or non-diabetic patients
- Patients had to have the pulpal diagnosis of normal pulp (elective treatment), reversible (elective treatment) or irreversible pulpitis

Exclusion criteria

Patients were ineligible for participation in the research study if they met the following criterion:

- Patients who knew that they were pregnant or may have been pregnant
- Pulp necrosis or previous endodontic treatment

PROCEDURES

Clinical Methodology

Patients were recruited from the standard referral base if pulpal diagnosis of any of the three categories of vital pulp status was established during the clinical and radiographic examination – normal pulp (elective treatment), reversible pulpitis (elective treatment), or irreversible pulpitis. Informed consent was reviewed and obtained for endodontic treatment, as well as to participate in the research study. Once endodontic treatment was

initiated under rubber dam isolation, a pulpal sample was retrieved from the largest root canal with a barbed broach. Irrigation was avoided during or after access to prevent diluting or contaminating the tissue sample. The pulp specimen was obtained and transferred to a 5ml EDTA vacutainer (Fig. 4) and stored in a -80° C freezer until needed for laboratory analysis. At the end of the endodontic treatment session, a peripheral blood sample was taken via venipuncture by one of three registered nurses in the dental school, at which time two 5ml vacutainers of blood were collected. One 5ml vacutainer of blood was submitted to the Faculty Physicians Inc Laboratory at the University of Maryland for HbA1c analysis only, and the second vacutainer was later analyzed in the McLenithan laboratory in the Division of Endocrinology, Diabetes and Nutrition, University of Maryland School of Medicine, for plasma AGE analysis. It was assumed that plasma AGE would serve as a control for pulpal AGE. Thus, both were measured and the correlation between pulpal AGE and plasma AGE was tested statistically. This protocol was followed, regardless of the reported diabetic status of the patient. No identifying information was provided other than the study coded name, study coded patient identification number, date of birth and gender (as required by the Laboratory).

Figure 4: Pulp sample under 10x magnification stereomicroscope

Laboratory Methodology

Pulp tissue has never been analyzed for AGE concentration. Therefore, a reliable laboratory methodology for its accurate analysis was the primary objective. An initial protocol was created, and was revised throughout a series of experiments. Cell lysates of the tissue were prepared for evaluation of AGE concentration, using the OxiSelect™ N^{ε}-(carboxymethyl)lysine (CML) Competitive ELISA Kit. CML values were normalized to total protein via the Extra Sense™ BCA Protein Assay Kit.

Developing a reliable and consistent laboratory methodology for measuring AGEs:

A total of six CML ELISAs were performed over an eight-month period. No previous report in the literature provided established procedures for preparing dental pulp tissue for CML analysis. Thus, the protocol that was created was as follows: (1) Specimens

were extracted (with microforceps, under stereomicroscope) from the barbed broach initially used to retrieve the tissue from the root canal system (Fig. 4). (9) Specimens were suspended in approximately 100-300µl buffer solution (depending on the size of the tissue sample). (3) Homogenization was performed via sonication for 30 seconds, followed by centrifugation for 2 minutes at 16,000xG.

Buffer

The Cell Biolabs, Inc ELISA kit stated that lysates could be prepared in any lysis buffer. The buffer that was originally selected for tissue suspension was radioimmunoprecipitation assay (11) buffer. This solution contains the ionic detergent, sodium deoxycholate, as the active constituent; is known to give low background, and has been used in several studies to evaluate tissue CML content (54, 55). In the first experiment, samples were diluted in RIPA (radio-immunoprecipitation assay) buffer at a 1:2 dilution. After performing the ELISA according to manufacturer's instructions, AGE values were normalized to overall protein content via the Extra Sense^{TM} BCA Protein Assay Kit. Results from this first experiment yielded extraordinarily high values, indicating that there might have been some interference from the buffer used. Thus, in subsequent experiments, 50mmol Hepes 0.5% Triton-X100 (Hepes Triton buffer), pH 7.4 was used to homogenize all pulp samples.

Protease Inhibitors

In the second experiment, two samples were divided in half, with one segment treated with Complete Protease Inhibitor cocktail from Roche to determine their necessity in

retarding digestion of the sample by natural proteases. No obvious differences were observed between split samples.

Red Blood Cell Lysis

An important consideration was blood contamination of tissue samples. Every specimen was retrieved from vital pulp tissue, however some samples were more saturated with heme than others. Two samples were split into two segments, and treated with or without red blood cell (RBC) lysis buffer in an attempt to reduce interference from CML-containing protein content in blood, which accompanied many samples. However, this proved to be ineffective as CML values from split samples appeared to be similar with or without the RBC lysis treatment. In addition, an important and beneficial rinsing step was incorporated into the laboratory protocol in an effort to reduce potential background. Specimens were rinsed with 1X PBS until the majority of the blood contamination was removed, and then suspended in Hepes-Triton buffer.

Proteinase K

Three samples were pretreated with Proteinase K in an attempt to yield greater AGE values. AGEs exist as modifications on proteins. Theoretically, antibodies may not be able to bind to all modifications due to steric hindrance. Thus, it was considered that the addition of an enzyme could aid in binding opportunity by separating the protein into multiple segments, eliminating steric hindrance and allowing adequate space for antibodies to bind to more modification sites. Proteinase K-treated samples were incubated for 3 hours at 37 ^{o}C with the enzyme, and then it was inactivated at 80^{o}C for 10

minutes (56) (57). One sample was split and diluted 1:2.5 in the proprietary assay diluent, as well as two different concentration of the Hepes-Triton buffer (1:2.5 and 1:5). Treated samples were then cooled to room temperature in preparation for plating. A microplate was precoated with an antibody specific for the AGE, N^{ε}-(carboxymethyl)lysine (CML) (Fig. 2), and the ELISA was performed according to manufacturer's instructions. All analyzed samples underwent the same laboratory protocol subsequent to method development.

Patients also received a standard HbA1c test, whereby a blood sample was drawn by one of the three registered nurses in the dental school. Each sample was submitted to the Faculty Physicians Inc Laboratory at the University of Maryland, for HbA1c analysis by a standard assay, in which blood samples were subjected to protease digestion with Bacillus *sp* protein, releasing amino acids, including glycated valines, from the hemoglobin beta chains. These glycated valines then become substrates for specific recombinant fructosyl valine oxidase (FVO) enzyme, which is produced by *E. coli (26).* In this assay, the FVO cleaves N-terminal valines, producing hydrogen peroxide, which is measured using a horseradish peroxidase (POD) catalyzed reaction and chromagen (26). HbA1c was then directly analyzed by the calibration curve.

RESULTS

Statistical Analysis

For H(iia and iib) and H(iva and ivb), Pearson's r were used. Since no significant correlations were found, the differences between iia and iib, and iva and ivb were not explored statistically. For H(iii), a 1-way ANOVA were used for statistical analysis. A $p \leq 0.05$ will be considered significant.

Primary Aim (i)

The result of the primary aim was to establish a reliable method by which AGEs could be measured in the pulp. The developed protocol was as follows:

Preparation of samples

a. Extract sample from barbed broach using microforceps under stereomicroscope
b. Rinse tissue with 1X PBS
c. Suspend pulp sample in 150µl cold Hepes-Triton buffer
d. Sonicate suspended tissue for 30 seconds
e. Centrifuge suspension for 2 minutes at 16,000xG
f. Remove supernatant for analysis

Analysis of CML content via ELISA

g. OxiSelectTM Nε-(carboxymethyl)lysine (CML) (Fig. 2) Competitive ELISA
h. The Secondary Antibody should be diluted at 1:2000 (instead of 1:1000) to slow the reaction with the primary antibody. The CellBiolabs OxiSelectTM Nε-(carboxymethyl)lysine (CML) (Fig. 2) Competitive ELISA kit states

that the reaction can take between 5-20 minutes, but it was observed, upon rapid colorimetric change, that the reaction took as little as 4 minutes. Thus, lowering the concentration of the secondary antibody proved to be effective in slowing down the reaction time.

Analysis of total protein content via Protein Assay

(1) Bicinchoninic acid (BCA) assay

Interpretation of CML concentration mathematically

(1) Divide total protein content by CML content

<u>Secondary Aims</u> (ii-iv)

Number of Subjects

The results of the power analysis required fifty-five subjects. However, the final number of participants was twenty-three. None of the enrolled subjects withdrew from the study. The final numbers were, for HbA1c, n = 23; pulpal AGE, n = 20; plasma AGE, n = 17, and for EPT, n = 23 (Table 1, Table 2). Two pulp samples were excluded due to insufficient tissue, and one pulp sample was excluded due to inadvertent fixation in formalin following retrieval, and was rendered unusable. Six plasma samples were excluded from analysis. The protocol was updated after the second subject enrolled, to include the acquisition of plasma. Therefore, plasma AGE values for the first two subjects were unavailable. Two samples were mishandled following retrieval, and the data for the final two samples were unavailable for analysis due to complications in method development.

Only one participant was a diagnosed diabetic; however there were four self-reported non-diabetics who had HbA1c values ≥ 6.5%. There were only three teeth with a pulpal diagnosis of normal pulp (non-inflamed), who required elective endodontic therapy.

Table 1: Data for Biomarkers

Biomarkers	n
HbA1c	23
Pulpal AGE	20
Plasma AGE	17
EPT	23

Table 2: Descriptive Statistics for Biomarkers

Biomarkers	Mean	Standard Deviation (SD)
HbA1c	5.57	.73
Pulpal AGE	4.42	5.95
Plasma AGE	8.74	4.83

Results of Aims iia and iib

There was no significant correlation found between pulpal AGE and HbA1c (r=-0.083, p=0.372). There was also no significant correlation found between plasma AGE and HbA1c (r=0.141, p=0.294, Table 3).

Table 3: Pearson's correlation of pulpal AGE, plasma AGE, EPT values and HbA1c

Biomarkers	HbA1c		
	r	r^2	p
Pulpal AGE	**-0.083**	**0.007**	**0.372**
Plasma AGE	**0.141**	**0.020**	**0.294**

Results of Aim iii

There was no significant difference in pulpal AGE values between inflamed and non-inflamed pulps (F=.148, p=.71). The mean ± standard deviation for pulpal AGE found in inflamed pulps (n=18) was 5.08 ± 5.80. The mean ± standard deviation for pulpal AGE found in non-inflamed pulps (n=2) was 3.46 ± .07 (Table 4).

Table 4: Comparison of pulpal AGE in inflamed vs. non-inflamed pulps

Groups	n	Mean ± SD	F	p value
Inflamed	18	5.08 ± 5.80	.148	.71
Non-inflamed	2	3.46 ± .07		

Table 5: Comparison of pulpal AGE in symptomatic vs. asymptomatic cases

Groups	n	Mean ± SD	F	p value
Symptomatic	11	3.21 ± 2.47	3.319	.087
Asymptomatic	7	8.01 ± 8.30		

Table 6: Pearson's correlation of EPT and pulpal AGE and EPT and plasma AGE

	EPT		
	r	**r^2**	**p**
Pulpal AGE	-.41	.17	.036
Plasma AGE	-.253	.06	.16

Results of Aim iv

There was a significant inverse correlation found between pulpal AGE and EPT values (r=-.41, p=.031) and a non-significant inverse correlation between plasma AGE and EPT (r=-.25, p=.16, Table 6).

DISCUSSION

The association between glycosylated proteins and diabetes was first proposed in the 1960s, with the discovery of an altered form of hemoglobin, HbA1c, in the red blood cells of patients with diabetes (58). Since then, numerous studies have investigated the association of AGEs with diabetic neuropathies, with reports of elevated AGEs in the serum plasma, saliva, and gingival crevicular fluid of patients with diabetes. At the start of this study, there was no published report measuring AGEs in human dental pulp. Thus, the primary aim was to develop a predictable and consistent laboratory protocol for measuring pulpal AGEs. Once this was established, associations between biomarkers were evaluated. Pregnant patients were not included in this study, because according to the University of Maryland Institutional Review Board ethical guidelines, they were considered to be a vulnerable population. One of the most important elements of the laboratory protocol was the addition of a rinsing step, whereby pulp samples were rinsed in 1X PBS prior to suspension in the Hepes-Triton buffer. The researchers were aware that hemolyzed plasma results in lysed RBCs, which may contribute to non-specific binding of proteins in the heme. This step resulted in purer samples, and more consistent pulpal AGE values.

Correlation between HbA1c and pulpal AGE

The results of this study did not reveal significant correlations between HbA1c and pulpal or plasma AGE. This is not surprising, given the fact that these hypotheses were based on a sample size derived from a power of .80. Fifty-five total subjects were needed to yield significant correlations between these biomarkers. It is likely that with an increased

number of subjects, the correlation between HbA1c and pulpal AGE, and between HbA1c and plasma AGE would become significant. Based on the findings of this study, the r^2 value indicated that HbA1c contributed only .7% to the pulpal AGE level. Furthermore, the r^2 value indicated that HbA1c only contributed 2% to the plasma AGE level. This degree of association is not considered clinically meaningful.

In addition, the researchers believe that, due to the inadequate sample size, much of the contribution of hyperglycemia (via HbA1c measurement) to the pulpal and plasma AGE levels could have been due to chance. However, replication of this study, with sufficient power, might yield lower standard deviations, and thus less variability. This then might yield significant relationships that would produce clinically meaningful results.

Differences in pulpal AGE in inflamed vs. non-inflamed pulps

The findings of this study did not indicate significant differences in pulpal AGE levels between inflamed and non-inflamed pulps. This is not surprising, given the low sample size and the discrepancy in the number of subjects between groups.

Differences in pulpal AGE in symptomatic and asymptomatic pulps

The majority of cases had a pulpal diagnosis of irreversible pulpitis (asymptomatic or symptomatic). Both were included in the inflamed category (n=18) while only two teeth had a normal pulpal diagnosis. An n of two in the non-inflamed group was much too small to allow for statistically and clinically meaningful results. The mean ± standard deviation in the inflamed group was 5.08 ± 5.80 and the mean ± standard deviation in

the non-inflamed groups was 3.46 ± .07 (Table 4). The large variability in the inflamed group may be attributed to the differences in blood contamination of pulp samples. Rinsing pulp samples with 1X PBS was incorporated into the protocol at a later stage, but was not performed in the initial experiments. Controlling for this issue in subsequent research may lower some of the variability observed in this study. In addition, both symptomatic and asymptomatic cases were included in the inflamed group which also may have had an effect on the observed variability, and subsequently contributed to the large standard deviation reported here.

There is currently no established value for pulpal AGE, therefore these AGE values should be considered with caution. For the inflamed group, the standard deviation was greater than the mean, a problem, and also may not be reliable given the fact that the n=55 was not achieved. For the non-inflamed group, the mean was lower than that of the inflamed group, which was expected because AGEs are increased in "stressful" environments, such as inflamed tissue (5). The standard deviation in the non-inflamed group was at an appropriate level in relation to the mean, unlike that of the inflamed group. However, since the n was only two, the researchers do not have confidence in these results. Caution must be used in interpretation of these values and replication with a larger sample size may yield different results.

A post hoc analysis was computed, based on these ANOVA results, to determine the number of subjects needed to yield a significant difference between the inflamed and non-inflamed groups. With an n of 184, a mean ± SD in the inflamed group of 5.08 ±

5.80 and 3.46 ± .07 in the non-inflamed group, and a $p \leq .05$, power was equal to .80. Even though the standard deviation was appropriately small in the non-inflamed group, the n of 184 was a result of the extremely large standard deviation in the inflamed group. True variability in pulpal AGE is expected in the inflamed group. Error variability in the data of this research is likely due to differences in blood contamination and the inclusion of symptomatic and asymptomatic pulpitis cases in the inflamed group. As the methodology for processing pulp tissue and analyzing pulpal AGE is improved, the error variability is expected to be reduced in the inflamed group. Subsequently, a lower n than 184 will be needed to yield significant differences between groups.

Research has yet to elucidate whether symptom status affects variability in pulpal AGE values. However, it is more likely that whether the patient is symptomatic or asymptomatic, there will be little observed differences between the groups. Seltzer et al discussed the histologic variability observed in symptomatic and asymptomatic patients. In asymptomatic or mildly symptomatic patients, the histologic conditions included intact uninflamed pulp, atrophic pulp, transitional stage, acute pulpitis, chronic partial pulpitis, hyperplastic pulpitis, and even pulp necrosis. Contrastingly, in patients with moderate-severe symptoms, the pulp was described as ranging (with less variety) from chronic partial pulpitis to pulp necrosis (59). It appears that in symptomatic patients, the pulpal status is more predictably inflamed than in asymptomatic cases, where the range of histologic conditions is vast. The association between pulpal AGEs in inflamed pulps of symptomatic vs. asymptomatic cases may become clearer in future studies with the addition of cold test and VAS responses.

In summary, the large standard deviation observed in the inflamed group is not likely due to the inclusion of asymptomatic and symptomatic cases, but rather due to issues in method development, with a resultant lowering of the n of 184 estimated in the post hoc power analysis.

EPT and pulpal AGE

The results of this study indicate a significant inverse relationship between EPT readings and pulpal AGE ($r=-.41$, $p=.036$). This is an interesting finding because the EPT is routinely used to assess pulp vitality (as simply present or absent), rather than to predict true pulpal status. In other words, EPT has not been established as a reliable predictor of the degree of pulpal inflammation. The functionality of the EPT is based on a threshold stimulus - a measure of the amount of provocation needed to evoke the lowest perception of pain (47). Intuitive interpretation would indicate that a lowered threshold would correspond to pulpal inflammation. Thus, lower EPT values would yield higher levels of inflammatory-related, glycated proteins (AGEs). However, due to the lack of literature supporting the distinct relationship between EPT results and degree of inflammation, our hypothesis was that there would be no significant correlation between EPT values and pulpal or plasma AGEs.

Early studies investigated the relationship between EPT values and the histological appearance of pulp tissue. According to Mumford, EPT results in vital teeth depended on the threshold at which the stimulus evoked a patient's response (pain threshold), whereas

it relied more heavily on electrical resistance in necrotic teeth, in the absence of a pain response. Electrical resistance is related to the amount of moisture in the tooth, and could conceivably give some indication of pulp status. It has been described that a low maximum current, associated with a high resistance, might represent a necrobiotic pulpal environment, whereas a high maximum current, associated with a low resistance, might signify a purulent or gangrenous pulpal condition (60). However, a variety of electrical stimulatory responses were reported to correspond to the same clinical diagnosis, and likewise considerable variations in the histological appearances of pulp tissue correspond to the similar EPT responses. Histological evaluation of teeth that responded to electrical stimuli, in Mumford's study were divided into three (3) categories: those with threshold stimuli in the normal range, those with a lower range, and those with a higher range. Results revealed that teeth with pain threshold values in the same range had varying histological pulp appearances, ranging from normal tissue, pulps with calcification, reticular or fibrous degeneration, acute pulpitis, mild chronic pulpitis, severe chronic pulpitis, acute abscess, and subtotal necrosis (60). Thus, this study did not support a correlation between the pain perception threshold and the histological state of the pulp.

In this study, all experimental teeth were clinically diagnosed as having vital pulps (as per the inclusion criteria), and the preoperative diagnosis was confirmed upon endodontic access. The majority of cases had inflamed vital pulps (n=20), with the lowest EPT reading being 21 and the highest reading 69, thus representing a high degree of variability in values within the same clinical diagnostic category of irreversible pulpitis. The three values in the non-inflamed group were 30, 79, and 41. Thus, the reported inverse

relationship between EPT and pulpal AGEs must be interpreted with caution. Pulpal inflammation and degeneration of the pulp is a process, which takes time to affect the entire tissue mass. Since the electric current passes through the entire length of the pulp tissue, if any vital tissue exists in the most apical part of the root canal system, it may be stimulated and subsequently elicit the patient's response (47). This would result in a low EPT reading, when in actuality, the majority of the pulp has degenerated. Furthermore, a histologically inflamed pulp may or may not be accompanied by clinical symptoms. However, the presence of inflammation might alter the threshold to noxious stimulation such that a small electrical stimulus might be sufficient to reach the pain threshold – a concept termed hyperalgesia (47). This is the reason it was suggested that there could be a relationship between pain threshold and pulp status. It was speculated that the threshold was lower in acute pulpitis including hyperemia, but higher in chronic pulpitis and in cases with tissue degeneration (47). However, the majority of the endodontic literature does not provide enough supportive evidence to support a distinct relationship between the pain perception threshold and the histological status of the pulp, namely due to the variability of histologic appearance in teeth with the same EPT responses, as well as varied EPT responses with similar histological pulp condition (47).

LIMITATIONS

The limitations of this study were as follows:

(1) Clinically meaningful relationships between biomarkers were unattainable due to insufficient power.

(2) Subsequent to the low sample size, there were too few samples to truly investigate the effect of Proteinase K on AGE content. The potential of an ELISA to detect AGE-

modified proteins may be reduced due to steric hindrance. Therefore proteinase K has been proposed as an adjunct for pretreatment of samples. The enzyme segments the protein, allowing for increased binding of antibody to modifications, thus increasing detection capability, and subsequent fluorescent signal. Proteinase K has been used for pretreatment of murine serum and spleen homogenates prior to CML analysis via ELISA (61).

FUTURE DIRECTION

(1) Replication of this study with sufficient number of subjects would be ideal. Now that the authors have determined that it is feasible to measure pulpal AGE, and have developed a consistent protocol by which to attain these measurements, future studies should focus on increasing power to evaluate significant relationships between biomarkers.

(2) Future research may include weighing pulp tissue as opposed to performing a protein assay to normalize AGE values to total protein. Pulp samples vary in size, which affects homogenization and AGE quantification. In this study, sonication was followed by centrifugation, yielding pellets, which may have retained connective tissue and subsequent AGEs. Pellet content was compared to supernatant content and found to be similar, which indicated successful homogenization. Therefore the researchers were confident in measuring only supernatants for pulpal AGE values. Weighing the pulp tissue before homogenization, and weighing the pellet after centrifugation may add to the accuracy in determining pulpal AGE content.

The proteinase K pretreatment method should also be explored. Fundamentally, this study was designed to elucidate a biological mechanism, of which hyperglycemia, inflammation, glycated proteins, and neuropathy are all involved and interrelated.

The design of future studies should translate this information to clinical observations. For example, one might design a longitudinal study, tracking the HbA1c in patients with diabetes who report symptoms of reversible pulpitis, and to monitor the progression of clinical pulp status. It would be interesting to observe the number of patients who develop irreversible pulpitis, necessitating endodontic therapy.

CONCLUSIONS

(1) The main finding in this study demonstrated the feasibility of measuring pulpal AGE from clinical samples of patients undergoing endodontic therapy.

(2) Pulpal AGE was significantly correlated to plasma AGE, which supports successful method development in detecting pulpal AGEs.

(3) There were no significant correlations between HbA1c and pulpal or plasma AGE. However, these results are likely due to insufficient power.

(4) There was a significant inverse correlation between EPT results and pulpal AGEs. These findings must be interpreted with caution because the endodontic literature does not support the EPT as a reliable predictor for level of pulpal inflammation at a histological level.

These findings hold great promise for pulpal AGE as a clinical biomarker. Its value lies in further elucidation of the mechanism underlying the relationship between hyperglycemia and pulpal neuropathy.

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