**In vivo astaxanthin treatment partially prevents antioxidant alterations in dental pulp from alloxan-induced diabetic rats**

**M. F. Leite, A. de Lima, M. M. Massuyama & R. Otton**
Ciências Biológicas e da Saúde, Universidade Cruzeiro do Sul – São Paulo, Brazil

**Abstract**

**Aim** To evaluate the effect of astaxanthin on antioxidant parameters of dental pulp from diabetic rats. The hypothesis tested was that supplementation of diabetic rats with astaxanthin might eliminate, or at least attenuate, the defect in their antioxidative status.

**Methodology** Wistar rats (*n* = 32) were divided into four groups: untreated control, treated control, untreated diabetic and treated diabetic rats. A prophylactic dose of astaxanthin (20 mg kg⁻¹ body weight) was administered daily by gavage for 30 days. On day 23, diabetes was induced by injection of alloxan (60 mg kg⁻¹ body weight). After 7 days of diabetes induction, the rats were killed, and pulp tissue from incisor teeth removed. Superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and reductase activities were determined. Data were compared by ANOVA and the Newman–Keuls test (*P* < 0.05).

**Results** Diabetes caused a reduction in SOD, GPx and reductase activity in dental pulp tissue. Astaxanthin had no effect on SOD and catalase activities; however, it stimulated GPx in control and diabetic rats.

**Conclusions** Diabetes altered the antioxidant system in dental pulp tissue; astaxanthin partially improved the diabetic complications.

**Keywords:** antioxidant system, astaxanthin, dental pulp, diabetes, oxidative stress.

**Introduction**
Diabetes Mellitus is a metabolic disease caused by impaired insulin secretion from pancreatic beta-cells and/or impaired insulin action in peripheral tissues, such as skeletal muscles, adipose tissue and liver (Araki & Miyazaki 2007). Diabetes is one of the components of metabolic syndrome regularly associated with cardiopathy, nephropathy, retinopathy and circulatory disease (Evangelista & McLaughlin 2009). As previously reported, the production of reactive oxygen species (ROS) is elevated in diabetes, particularly amongst those who have poor glycemic control, and it has been hypothesized that increased ROS generation in long-standing diabetes might result in oxidative damage to DNA (Wyatt *et al.* 2006). Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids and eventually lead to cell death, events related to late diabetic complications (Maritim *et al.* 2003).

Patients with diabetes present with oral manifestations such as xerostomia, taste impairment and salivary changes (Bernardi *et al.* 2007) that might affect the progression of certain diseases, such as dental caries, periodontal disease, soft tissue lesions and fungal infections (Manfredi *et al.* 2004). The literature on the pathogenesis, progression and healing of endodontic pathosis in patients with diabetes is scarce. A review of diabetes as a modulating factor of endodontic infections revealed an increased prevalence of root filled teeth.
with periradicular lesions and a disproportionately high percentage of clinically severe pulpal infections, suggesting some differences in the natural history of endodontic diseases in patients with diabetes (Fouad 2003). Alterations have been observed in dental pulp associated with diabetes, including: circadian rhythms of pulp sensitivity, which differ from those of healthy people (Guo et al. 2007); decreased retention of root filled teeth and increased incidence of retreatment (Mindiola et al. 2006); and a reduced likelihood of successful root canal treatment in cases with preoperative periradicular lesions (Fouad & Burleson 2003). Moreover, diabetes inhibited dentine bridge formation in teeth with exposed pulps in rats (Garber et al. 2009).

Dental pulp is a metabolically active connective tissue specialized in the production of dentine and possesses a high capacity for regeneration and turnover (Goldberg et al. 2008). Dental pulp tissue is enclosed within the rigid structure of the mineralized tissues of the tooth and requires active drainage of the extracellular matrix to eliminate excess fluid and macromolecular compounds. This tissue has an aerobic metabolism, especially during tooth development, whilst its vitality is related to blood circulation and oxygenation. Thus, the equilibrium between antioxidant parameters helps to prevent damage caused by oxidative products generated in tissues under aerobic metabolism. In addition, the activity of antioxidant enzymes has been described in association with several biological conditions in the dental pulp (Tulunoglu et al. 1998, Esposito et al. 2003).

Considerable evidence exists that the hyperglycaemia observed in diabetes results in the generation of ROS (glucose oxidation is believed to be the main source of free radicals), ultimately leading to oxidative stress in a variety of tissues. In particular, diabetes mellitus is strongly associated with oxidative stress, which could be a consequence of increased free radical production, reduced antioxidant defences or both. Several mechanisms have been suggested to contribute to the formation of these reactive oxygen-free radicals in diabetes. In fact, a major source of reactive molecules, including superoxide and hydrogen peroxide (H2O2), is the enzyme NAD(P)H oxidase, which is activated by a variety of inflammatory cytokines (Evans et al. 2005). Imbalance in the antioxidant system is a condition that has been frequently reported in the literature associated with diabetes (Maritim et al. 2003).

Hyperglycaemia induces overproduction of superoxide that contributes to the pathogenesis of micro- and macrovascular diabetic complications (Rolo & Palmeira 2006). Vascular changes could cause irreversible damage in the dental pulp. Biological organisms possess an enzymatic system of defence against oxidative damage, mainly composed of superoxide dismutase (SOD), catalase, the glutathione system (peroxidase and reductase) including dental pulp (Bodor et al. 2007, Lee et al. 2008). The secondary defence is the nonenzymatic system, most of which are dietary derivatives, such as vitamins C, E and carotenoids (Gate et al. 1999).

Several antioxidant therapies using different carotenoids have achieved success in the treatment of complications caused by diabetes (Ali & Agha 2009). Astaxanthin is a carotenoid pigment found naturally in certain fish, birds, crustaceans and microorganisms. Humans and others animals are unable to manufacture carotenoids and hence require these in their diet. Because of pharmaceutical and food demand, astaxanthin is widely used in the form obtained from the fermentation process of green microalgae or red yeast. Some benefits of astaxanthin have been reported, including anticancer properties, enhanced cell-mediated immune response, preservation of the beta-cell function, protection of human lens epithelial cells against UV-B insult and attenuated exercise-induced damage in mouse skeletal muscle and heart (Higuera-Ciapara et al. 2006, Hussein et al. 2006, 2007). The main tissues showing benefits with the administration of astaxanthin are cardiovascular, cardiac and skeletal muscle, vasculature, ischaemic brain and kidney (Fasset & Coombes 2009).

The biological potential of astaxanthin is related to its antioxidant capacity, particularly in diabetes (Uchiyama et al. 2002, Manabe et al. 2008). Astaxanthin contains two additional oxygenated groups on each ring structure, resulting in enhanced antioxidant properties. Astaxanthin can minimize the effect of peroxyl and superoxide radicals and singlet oxygen and protects membranes from lipid peroxidation associated with diabetes (Fasset & Coombes 2009). Some reports have shown that retinoic acid contributes to dentine mineralization by up-regulating dentine sialoprotein expression in dental pulp (Ritchie et al. 2004); however, there is a lack of reports regarding the effect of astaxanthin in this tissue.

It was previously shown that dental pulp from diabetic rats stimulated catalase activity, suggesting an increase in oxidative stress in the dental pulp tissue of diabetic rats (Leite et al. 2008). The oxidative stress could cause damage to biomolecules such as DNA, proteins and lipids, compromising the functions of dental pulp. Astaxanthin can be an adjunct in the treatment of diabetes, because it might restore some
important cellular functions or at least prevent oxidative damage caused by a ROS-overproduction. Considering the excessive generation of ROS in diabetes mellitus, it has been proposed that the supplementation of diabetic rats with astaxanthin might antagonize, or at least improve, the defect in their antioxidative status. Thus, the purpose of this study was to evaluate the effect of astaxanthin on the antioxidant enzymes of dental pulp from alloxan-induced diabetic rats.

Materials and methods

Reagents

Astaxanthin, alloxan monohydrate, nitroblue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced nicotinamide adenine dinucleotide (NADH), Triton X-100, 2,4-ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), reduced glutathione (GSH), oxidized glutathione (GSSG), hydrogen peroxide (H₂O₂) and streptomycin were all purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Other reagents for buffers (e.g. PBS) and regular laboratory solutions were obtained from Labsynth (Diadema, São Paulo, Brazil).

Animals and astaxanthin treatment

Male Wistar rats (n = 32), weighing 50 ± 10 g at the onset of the study, were obtained from the Butantan Institute, São Paulo, Brazil. The rats were fed ad libitum and maintained in a room at 23 °C and lights on from 07:00 to 19:00. The Ethics Committee of the Cruzeiro do Sul University, São Paulo, granted ethical approval (nº030/07). After an acclimatization period of 1 week, Wistar rats were treated by gavage 5 days a week for 30 days with astaxanthin at the dose of 20 mg kg⁻¹ of body weight using olive oil as a vehicle. The rats were separated into four groups, each composed of ten rats. The groups were designated as follows: (i) untreated control (UC), the group only received olive oil by gavage throughout the experiment; (ii) treated control (TC), the group was astaxanthin-treated (20 mg kg⁻¹ of body weight) by gavage throughout the 30-day period; (iii) untreated diabetic (UD), the group was alloxan-treated to induce diabetes 23 days after the onset of the study; and (iv) treated diabetic (TD), alloxan injection was used to induce diabetes after 23 days of astaxanthin treatment. This group continued receiving astaxanthin for the final 7 days of the 30-day astaxanthin treatment period.

Induction of diabetes

The experimental and control rats underwent fasting for 12 h prior to alloxan administration. Freshly prepared alloxan in PBS (0.137 mol L⁻¹ NaCl, 2.7 mmol L⁻¹ KCl, 8.0 mmol L⁻¹ Na₂HPO₄, pH 7.4) was injected intraperitoneally at a dose of 75 mg kg⁻¹ of body weight. Blood sugar was estimated once, 24 h after alloxan administration, using a glucometer to confirm the development of diabetes mellitus. Blood glucose was determined at the time of killing in the morning, using a kit from Bioclin (São Paulo, Brazil) and following manufacturers’ instructions. The rats were not fasting. These rats showed weight loss (because of uncontrolled diabetes) and severe hyperglycaemia. The diabetic rats used in this study were those presenting glycemia over 200 mg dL⁻¹, after 7 days of alloxan injection. The model of alloxan-induced diabetes was chosen because alloxan-diabetic rats present low plasma levels of insulin with no change in the activation state of the immune cells (Otton et al. 2002). Experimental procedure

Diabetic rats and matching controls were maintained under similar conditions. After 7 days of diabetes induction and 30 days of astaxanthin treatment, fed rats were killed by decapitation between 08:00 and 11:00. The heads were immediately removed and stored at −80 °C until required. Maxillary and mandibular incisor dental pulps were removed using Hedström files. Each sample represented a pool of four incisor pulps from each rat to obtain an adequate amount of tissue.

Measurement of antioxidant enzymes

Preparation of homogenates

The samples (± 50 mg) were homogenized at 10% with 0.5 mL of 50 mmol L⁻¹ sodium phosphate buffer, pH 7.4, vortexed briefly and rupted by ultrasonication in a Vibra Cell apparatus (Danbury, CT, USA) as previously described (Otton et al. 1998). A centrifugation step was included (10 000 g for 10 min at 4 °C) to eliminate debris from the crude homogenate, resulting in the precipitate and supernatant parts; the supernatant was then used for further analysis.

Assay of superoxide dismutase activity

The activity of SOD was measured (Ewing & Janero 1995), with minor modifications. The complete reaction buffer for total SOD included 50 mmol L⁻¹ sodium phosphate buffer, pH 7.4, 0.1 mmol L⁻¹ EDTA.
50 μmol L−1 NBT, 78 μmol L−1 NADH and 3.3 μmol L−1 phenazine methosulphate (PMS) used as an O2·− generator. The kinetic absorbance variation at 560 nm was continuously monitored for 2 min to evaluate O2·−-dependent reduction of NBT. A control system lacking PMS revealed negligible change in absorbance at 560 nm.

Assay of catalase activity
The decomposition of H2O2 can be followed directly by the decrease in absorbance at 240 nm (ε240 = 0.0394 ± 0.0002 L per mmol L−1 per cm−1). One catalase unit is defined as the enzyme concentration required for the decomposition of 1 μmol of H2O2 per min at 25 °C. All assay solutions were prepared at room temperature, as described by Aebi (1984). The complete reaction system for catalase consisted of 0.1 mmol L−1 phosphate buffer, pH 7.4, and 10 mmol L−1 H2O2. The reaction was initiated by the addition of 10 mmol L−1 H2O2, and absorbance was monitored for 2 min at 240 nm.

Assay of glutathione peroxidase activity (GPx)
GPx activity was measured according to the method described by Mannervik (1985). Enzyme activity was determined using 2.5 U mL−1 of glutathione reductase (GR), 10 mmol L−1 GSH, 250 μmol L−1 sodium azide (as a catalase inhibitor) and 1.2 mmol L−1 NADPH in the presence of 4.8 mmol L−1 tert-buthyl hydroperoxide used as substrate in a cell homogenate. The oxidation of NADPH was monitored at 340 nm for 2 min in 0.2 mol L−1 phosphate buffer, pH 7.4, in a Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Little Chalfont, UK).

Assay of glutathione reductase activity
Glutathione reductase activity was measured using the same methodology described by Mannervik (1985). Alternatively, GR activity was determined using 3.6 mmol L−1 NADPH and 10 mmol L−1 GSSG. Again, the NADPH oxidation was monitored in 0.2 mol L−1 phosphate buffer, pH 7.4, at 340 nm for 2 min.

Protein measurement
Specific enzyme activities were all related to protein concentrations, which were estimated by the Bradford method (Bradford 1976) using BSA as a standard.

Statistical analysis
The data are presented as mean ± standard error of the mean (SEM). The Anderson–Darling test was applied for the evaluation of the frequency distribution of the data. After checking the normality of data distribution, the biochemical parameters of the groups studied were compared by Analysis of Variance and the Newman–Keuls test. The level of significance adopted was 5% (P < 0.05).

Results
The blood glucose level (mg dL−1) was significantly higher (P < 0.05) in UD and TD groups (626.7 ± 40.8 and 607.8 ± 43.9, respectively) compared to the untreated and TC group (170.3 ± 16.6 and 172.2 ± 6.6, respectively). The astaxanthin presented no hypoglycaemic effects in the groups studied.

The results of the study are presented in Figs 1, 2, 3 and 4. Figure 1 presents the SOD activity (U mg protein−1) of dental pulp tissue from the groups studied. Diabetes caused a reduction (26%) in SOD activity (19.9 ± 3.6) compared to the UC rats (26.9 ± 2.3; P < 0.05). Treatment with astaxanthin had no effect on the SOD activity of the TD group; however, it promoted a reduction in this parameter in the TC group (26%; 19.8 ± 2.3; P < 0.05). No difference in the catalase activity of dental pulp was observed amongst the groups studied, according to results presented in Fig. 2.

The results of the GPx and GR activity (mU mg protein−1) of dental pulp from the groups studied are presented in Figs 3 and 4, respectively. A reduction of...
35% in GPx activity of the dental pulp from diabetic rats (105.71 ± 5.6) was observed compared to the UC group (160.47 ± 14.2; P < 0.05). Astaxanthin treatment had a stimulatory effect on GPx in both the TC (26%; 202.28 ± 22.3) and TD groups (40%; 149.40 ± 8.9; P < 0.05), antagonizing the alterations provoked by diabetes. The results of GR were similar to GPx, presenting a reduction in enzymatic activity in the UD group (25%; 137.78 ± 10.2) compared to the UC group (181.11 ± 16.3; P < 0.05). However, astaxanthin treatment did not prevent previous alterations observed in GR activity.

**Discussion**

The oral implications of diabetic patients have been the focus of interest of research in dentistry, especially through the use of therapies that could minimize the effects of this disease. In this study, a possible prophylactic role of astaxanthin, a carotenoid considered a potent antioxidant agent, was evaluated in dental pulp tissue from alloxan-induced diabetic rats, to investigate whether astaxanthin could antagonize dental pulp damage caused by diabetes. Analysis of the results showed that, even with the short diabetes induction period of 7 days, a reduction occurred in SOD, GPx and GR activity in dental pulp tissue from rats. Astaxanthin produced a stimulatory effect on GPx activity in both the TC and TD groups, achieving values similar to the UC group.

Under normal metabolic conditions, the production of free radicals and the antioxidant capacity are balanced. These ROS have an important biological role, including action on the immune system and control of vasodilatation and vasoconstriction, but in excess, they can be harmful to biological tissues. The antioxidant system is composed of a group of enzymes responsible for the control of free radicals and to minimize adverse cellular effects resulting from...
excessive exposure to reactive oxygen. SOD is an enzyme that depends on cofactors to control superoxide anions by converting them into hydrogen peroxide and oxygen. Catalase and GPx are enzymes responsible for hydrogen peroxide degradation into water and oxygen, reducing the toxicity of this compound in the tissues. In this reaction, the GPx oxidizes the thiol-GSH, donating an electron to hydrogen peroxide. The GSSG is reconverted to reduced glutathione by the action of the enzyme GR (Mates 2000). Considerable evidence exists confirming that an imbalance in the antioxidant system is associated with diabetes in humans and in animal models (Araki & Miyazaki 2007).

Superoxide dismutase activity has been studied in dental pulp tissue under inflammatory conditions; however, the results were contradictory. There are reports showing an increase in SOD in cases of irreversible pulpitis (Bodor et al. 2007) and reduction in its activity in irreversible symptomatic pulpitis tissue (Varvara et al. 2005). No reports exist regarding SOD activity in dental pulp from diabetic animals or human patients. The present results show for the first time that diabetes reduces SOD activity in dental pulp tissue from diabetic rats, corroborating the effect of the disease in other tissues (Fujita et al. 2009).

One report shows that diabetes increases catalase activity and reduces the concentration of others compounds that might act as hydrogen peroxide scavengers in dental pulp from streptozotocin (STZ)-induced diabetic rats (Leite et al. 2008). In this study, the short period of diabetes induction (1 week) revealed no effect on catalase activity in dental pulp tissue from diabetic rats. The differences in these studies could be explained by divergent experimental conditions, such as the type of diabetes induction (alloxan or STZ) and the time course of the disease. Although diabetes had no effect on catalase activity in the dental pulp, the glutathione system (GPx and GR activities) was reduced significantly. Thus, this reduction could contribute to the accumulation of hydrogen peroxide and tissue toxicity. Moreover, the imbalance in the glutathione system could affect the level of GSH, a compound with important biological functions including cell differentiation, proliferation, apoptosis, immune system activation and action in several human diseases (Ballatori et al. 2009). There is no report in literature relating the effect of diabetes on the glutathione system of dental pulp; however, some evidence shows that GPx could protect this tissue against mechanical stress (Lee et al. 2008).

It is accepted (Node & Inoue 2009) that endothelial dysfunction precedes and might cause diabetic microangiopathy. Reports have also revealed the role of oxidative stress in increased endothelial dysfunction (Szasa et al. 2007). The microvascular arrangement of the dental pulp plays a major role in hard- and soft-tissue physiology, and alterations of endothelial structure and function might also result in a pathological state (Trubiani et al. 2003). Diabetes causes a reduction in pulpal blood flow in STZ-induced diabetic rats (Amatyakul et al. 2006). The reduction in antioxidant parameters observed in this study could contribute to the oxidative damage in the microvascular circulation of pulpal tissue.

The development of strategies in vital pulp therapy, which aim to maintain the vitality and function of the dentine–pulp complex, represents a major focus of attention. One report affirms that a long-term supplementation of vitamin C prevents the reduction of pulpal blood flow caused by diabetes (Amatyakul et al. 2003). Retinoic acid promotes growth and differentiation in many types of cells, and its application in dental pulp cells increases alkaline phosphatase activity, a plasma membrane–bound enzyme that is generally involved in the mineralization process (San Miguel et al. 1999). The topical application of catalase in dental pulps might act as a direct capping agent, inducing a slight inflammatory cell infiltrate after exposure of the dental pulp, with a normal soft tissue response after 90 days (Ałaçam et al. 2000).

Antioxidant therapy has shown positive effects, inhibiting the progression of late diabetic complications, including polyneuropathy, cardiac autonomic neuropathy, micro- and macroangiopathy. Astaxanthin prevents the progression of diabetic nephropathy, protects the structure of the cellular membrane against lipid peroxidation (McNulty et al. 2008) and reduces glucose toxicity and the destruction of pancreatic β-cells. The protective action of astaxanthin in the treatment of diabetes is related to its ability as a ROS scavenger of singlet oxygen, superoxide radical and peroxyl radical species involved in tissue damage (Nakano et al. 2008).

The oral prophylactic application of astaxanthin stimulated GPx activity in dental pulp from healthy and diabetic rats. This enzyme has an important role in the control of hydrogen peroxide and other hydroperoxides, using the thiol-GSH as a donor electron to hydrogen peroxide. The reduction in GSH is frequently reported in diabetes disease, and the astaxanthin prevents the degradation of this molecule and maintains its availability for the GPx activity. The increased availability of GSH and GPx activity in dental pulp...
tissue could provide benefits associated with biological functions of this molecule, such as regulation of protein and gene expression, induction of apoptosis in transformed cells lines under stress condition and improvement in cellular immunity (Townsend et al. 2003). Unfortunately, astaxanthin prevents the degradation of GSH but does not stimulate its formation through the enzymatic activity of GR.

Besides direct prevention against oxidative damage caused by diabetes, the use of astaxanthin could prevent possible dental pulp damage caused by oxidizing agents, for example hydrogen peroxide used for dental bleaching. Astaxanthin improves the rheological properties of blood (Miyawaki et al. 2008), which could be an excellent form of prevention of microvascular disease in diabetic dental pulp. Moreover, studies of carotenoid astaxanthin and its derivatives demonstrated anti-inflammatory properties and potential efficacy in the setting of ischaemia-reperfusion (Bansal et al. 2006), a possible alternative for dental trauma and inflammatory disease of dental pulp.

Despite the limitations of this study performed on an animal model of diabetes, the results open a field of research concerning alternative therapies that could minimize the effects of diabetes on dental pulp. Astaxanthin-antioxidant therapy could maintain the redox homoeostasis in dental pulp of diabetic rats, a factor important to the vitality of this tissue. Further studies, including additional parameters, are required, especially works involving human tissues that lead to more definitive conclusions regarding oxidative damage to dental pulp under diabetic conditions and their clinical implications.

Conclusion

Diabetes conditions altered the antioxidant system in dental pulp, although astaxanthin partially improved the diabetic complications.

Acknowledgement

The authors are indebted to RCS Macedo, AP Bolin, C Jr Fineto, TP Geraldo. The present study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP process no.: 07/03334-6).

References


