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Elevated expression of Cu, Zn-SOD and Mn-SOD mRNA in inflamed dental pulp tissue

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Abstract

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Aim To determine the mRNA expression levels of copper-zinc superoxide dismutase (Cu, Zn-SOD) and manganese SOD (Mn-SOD) in healthy and inflamed human dental pulp tissue.

Methodology Sixteen patients with symptomatic irreversible pulpitis (eight females and eight males) were selected for study. Normal healthy pulps were removed from extracted mandibular third molar teeth from 10 systemically healthy individuals (six females and four males). QRT-PCR analysis of Cu, Zn-SOD and Mn-SOD mRNA expression was carried out in 16 cases of irreversible pulpitis and in 10 cases of systemically healthy donors. The Shapiro–Wilk's test was used to test the normality of data, whereas the Mann–Whitney *U*-test was used to evaluate the significance of the differences between groups. Differences in the expression levels were considered to be statistically significant for *P*-values <0.05.

Results A significant increase ($P < 0.05$) occurred in both Cu, Zn-SOD and Mn-SOD mRNA expression in cases of irreversible pulpitis. The increase in Mn-SOD level was significantly higher ($P < 0.05$) than the change observed for Cu, Zn-SOD.

Conclusions The development of pulpitis is associated with elevated transcription of both Cu, Zn-SOD and Mn-SOD; pulp tissue inflammation generated higher Mn-SOD transcription compared with Cu, Zn-SOD.

Keywords: inflammation, irreversible pulpitis, superoxide dismutase.

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Introduction

Reactive oxygen intermediates (ROIs) are a group of oxidative molecules released by host cells during the inflammatory processes (Freeman & Crapo 1982, Babior 1984). These molecules are also produced in relatively small amounts in healthy cells by incomplete reduction of oxygen in the mitochondrial matrix (Chance *et al.* 1979, Boveris 1984). Excess production of ROIs contributes to the pathogenesis of many diseases involving inflammation (Nussler & Billiar 1993) and represents a state in which tissues are susceptible to oxidative damage (Cerutti 1985).

To avoid excessive oxidative tissue damage caused by the cumulative load of ROIs, the concentrations of ROIs are controlled via various cellular defence mechanisms consisting of several antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutases (SODs) (Freeman & Crapo 1982, Halliwell 1999). SODs are detoxifying antioxidant enzymes which act against free radicals by catalyzing the dismutation of the superoxide ion into oxygen and hydrogen peroxide (McCord & Fridovich 1969). To date, two distinct intracellular SOD isoenzymes have been described in vertebrates, which differ by the type of metal in the active site of the enzyme and their intracellular localizations (Fridovich 1995). The copper-zinc SOD (Cu, Zn-SOD) has mainly been

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demonstrated in the cytoplasm (McCord & Fridovich 1969), and the manganese SOD (Mn-SOD) is found primarily in the mitochondrial matrix (Weisiger & Fridovich 1973).

Cu, Zn-SOD activity has been reported in human dental pulp (Grossi *et al.* 1991), but conflicting results were published concerning the activity of this enzyme in inflamed dental pulp. Tulunoglu *et al.* (1998) found no differences in total SOD activities between irreversible pulpitis and healthy pulp in children. In contrast to this finding, a significant increase of Cu, Zn-SOD enzyme activity was demonstrated in irreversible pulpitis in adults (Davis *et al.* 1991), suggesting that antioxidant defence mechanisms are active in the development of dental pulp inflammation. This finding was also supported by the increased immunoreactivity of both Cu, Zn-SOD and Mn-SOD in inflamed pulp using immunohistochemistry (Baumgardner & Sulfaro 2001). In contrast to these observations, most recently Varvara *et al.* (2005) reported a significantly lower Cu, Zn-SOD activity in irreversible pulpitis compared with the healthy dental pulp. The reduced Cu, Zn-SOD activity was interpreted as a result of depletion and destruction of the enzymes by the inflammation.

Based on these observations it is difficult to draw a definite conclusion whether increased, reduced or even unaltered SOD activity is associated with dental pulp inflammation. The different findings of different studies may originate from several reasons, including sampling of the tissue and the methods that investigators used for the evaluation of SOD activity. As in spectrophotometry, the most frequent method used in these studies, the calculation of SOD activity is based on the volume or weight of the pulp tissue, the oedema fluid and/or bleeding of the pulp tissue highly influence the measured enzyme activity (Sun & Zigman 1978). Furthermore, different pH conditions were used in different studies of SOD enzyme activity, which can also explain the discrepancy found in enzyme activity and consequently the results of the studies (Davis *et al.* 1991, Marklund 1992, Varvara *et al.* 2005).

To eliminate these confounding factors and to determine the SOD enzyme level in inflamed and healthy dental pulp, a more sensitive quantitative real-time polymerase chain reaction assay (QRT-PCR) was used, which measures levels of mRNA and is not influenced by pH found in tissues. Using this sequence-specific method, the Cu, Zn-SOD and Mn-SOD expression levels were analysed separately in inflamed and healthy dental pulp tissues.

Materials and methods

Patients and pulp tissue samples

All subjects gave informed consent to the scientific use of their specimens and research was conducted according to the highest principles of human subject welfare. Normal healthy pulps of extracted mandibular wisdom teeth were removed from 10 systemically healthy individuals (six females and four males). All teeth in this group were fully erupted, asymptomatic, caries free and intact. Sixteen patients with symptomatic irreversible pulpitis (eight females and eight males) were selected. These patients reported spontaneous pain and/or prolonged episodes of pain caused by sudden temperature changes. The pulp status of affected teeth was examined by using thermal testing. The aetiology of pulpitis was caries confirmed by the dental history, clinical and radiographic examination. Teeth with loss of lamina dura and with large, visible pulpal exposures were excluded. None of the patients used antibiotics 1 month prior to the study, and no long-term treatment with anti-inflammatory drugs was observed. The age distribution of patients is summarized in Table 1. Immediately after extraction, the teeth were longitudinally grooved and split in half with a chisel. The pulp chambers were opened using a slow-speed drill and pulp tissue samples were collected with a spoon excavator and reamers. After pulp removal the tissue was immediately placed into stabilizing RNA later solution (Qiagen, Hilden, Germany) to avoid RNA degradation. Samples were then stored at −80 °C until RNA isolation.

Quantitative real-time polymerase chain reaction analysis of Cu, Zn-SOD and Mn-SOD mRNA expression

Quantitative real-time polymerase chain reaction analysis of Cu, Zn-SOD and Mn-SOD mRNA expression was carried out in 16 cases of irreversible pulpitis and in 10 cases of systemically healthy donors. Total RNAs were extracted using Trizol reagent (Invitrogen, Carlsbad,

Table 1 Age of patients

Group	*n*	Age range (years)	Mean age (years) ± SE[a]
Donors of healthy pulp	10	18–74	34.3 ± 5.07
Donors of inflamed pulp	16	21–79	32.7 ± 4.10

[a]SE, standard error.

CA, USA) as recommended by the manufacturer. One microgram of RNAs was reverse transcribed to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The QRT-PCR assay was performed with ABI Prism® 7300 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). For amplification of Cu-Zn SOD and Mn SOD mRNA a TaqMan® based Assays-on-Demand™ Gene Expression product (Applied Biosystems) was used. To normalize the SOD values, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using a pre-developed TaqMan® Control Reagent (Applied Biosystems). All samples were run in triplicate, in a 20 μL reaction volume that contained 100 ng of cDNA. Sequence Detection Software version 1.3 (Applied Biosystems) was used to analyse the data after amplification. Results were obtained as threshold cycle (C_T) values. C_T represents the cycle number at which fluorescence passes a fixed threshold. Expression levels were calculated using the $\Delta\Delta C_T$ method. The values were calculated as the mean values of three independent measurements, and the expression levels of SOD mRNA in all samples were defined as the ratio of SOD to GAPDH expression.

Statistical analysis

The values of Cu, Zn-SOD and Mn-SOD mRNA expression were analysed using Statistica 6.0 software (StatSoft, Tulsa, OK, USA). The Shapiro–Wilk's test was used to test the normality of data, whereas Mann–Whitney *U*-test was used to evaluate the significance of the differences between groups. Differences in the expression levels were considered to be statistically significant for *P*-values <0.05.

Results

To determine the expression level of Cu, Zn-SOD and Mn-SOD mRNA, QRT-PCR assays were performed in 16 cases of irreversible pulpitis. The Cu, Zn-SOD and Mn-SOD mRNA levels of 10 healthy pulps were used as controls. The results of SOD mRNA expression are reported in Table 2 and shown in Fig. 1 as relative expression values. An increased Cu, Zn-SOD and Mn-SOD mRNA level in most cases of irreversible pulpitis was observed. In the irreversible pulpitis group, both SODs showed significantly higher expression compared with the healthy pulps ($P = 0.002766$ for Cu, Zn-SOD and $P = 0.000007$ for Mn-SOD). In cases of irreversible pulpitis, Cu, Zn-SOD mRNA levels were 5.16-fold (±1.70), and Mn-SOD mRNA level was 15.42-fold higher (±4.36) compared with normal controls (Fig. 1). In the normal pulp tissues, expression levels of Cu, Zn-SOD and Mn-SOD were not significantly different ($P = 0.911797$). In cases with irreversible pulpitis, the expression of Mn-SOD mRNA was significantly higher compared with the expression of Cu, Zn-SOD ($P = 0.018931$) (Fig. 1).

Discussion

To determine the expression levels of Cu, Zn-SOD and Mn-SOD mRNA in symptomatic irreversible pulpitis and to compare the expression levels of these enzymes to levels expressed in healthy dental pulp, a TaqMan

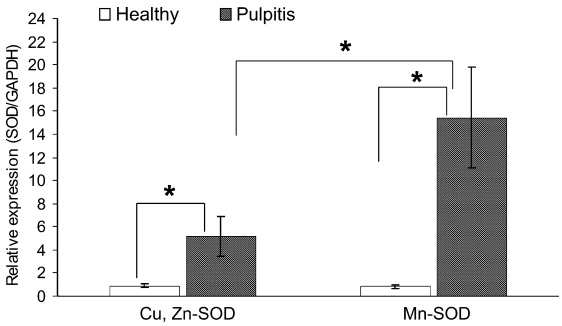

Figure 1 Relative expression levels of Cu, Zn-SOD and Mn-SOD mRNA in healthy pulp and inflamed pulp tissue, measured by QRT-PCR. *Statistically significant difference ($P < 0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; QRT-PCR, quantitative real-time polymerase chain reaction; SOD, superoxide dismutase.

Table 2 Relative expression levels of Cu, Zn-SOD and Mn-SOD mRNAs

	Cu, Zn-SOD		Mn-SOD	
Group	Mean (range)	SE[a]	Mean (range)	SE[a]
Healthy pulp	0.91 (0.15–1.99)	0.19	0.85 (0.2–2.79)	0.16
Inflamed pulp	5.16 (0.38–24.93)	1.70	15.42 (0.98–59.41)	4.36

[a]SE, standard error.
Cu, Zn-SOD, copper-zinc superoxide dismutase.
Mn-SOD, manganese superoxide dismutase.

based QRT-PCR approach was employed. The TaqMan based technology is an absolutely specific and highly reproducible system for detecting our target genes, as signal is only detected if the target sequence is complementary to the probe used (Heid *et al.* 1996). In this study, the TaqMan probes were designed to flank exon-exon boundaries (exon 1–2 for Cu, Zn-SOD; exon 4–5 for Mn-SOD), which rules out the possibility of genomic DNA amplification. The compensation for RNA degradation and pipetting errors was done by normalization of the target mRNA expression levels to a constantly expressed housekeeping gene (GAPDH). Therefore, the QRT-PCR assay provided highly accurate and specific quantitative results.

Using this technology a significantly higher Cu, Zn-SOD and Mn-SOD mRNA expression in the irreversible pulpitis cases compared with the noninflamed pulp tissue was found. Although, RNA level based observations are not always directly comparable with observations based on protein levels, such as measurement of enzymatic activity or immunoreactivity, these results are in agreement with previous studies demonstrating higher Cu, Zn-SOD and/or Mn-SOD enzyme activity or immunoreactivity in inflamed pulp compared with the normal pulp (Davis *et al.* 1991, Baumgardner & Sulfaro 2001). The findings are highly consistent with the theory that an active defence system is triggered against H_2O_2 during the inflammatory process in dental pulp tissue, and inflammatory reaction of dental pulp has similar mechanisms to that of inflammation of other tissues in the body (Forman & Torres 2001).

Previous studies mostly analysed total SOD and/or Cu, Zn-SOD activities in dental pulp tissue. Using the QRT-PCR technology the Cu, Zn-SOD and Mn-SOD mRNA levels were analysed separately. The results of this study provide evidence that in the inflammatory defence mechanism of dental pulp both SOD isoenzymes, the cytoplasmic Cu, Zn-SOD and the mitochondrial Mn-SOD are involved, although a significantly higher expression of Mn-SOD compared with the Cu, Zn-SOD was detected. The different expression of these two SOD isoenzymes can be a unique feature of dental pulp inflammation. Several lines of evidence suggest that the release of different cytokines and chemokines (interleukin-1B, interleukin-6, bradykinin) affects the activity and enzyme production of host cells in the inflamed tissue (Hosoya & Matsushima 1997, Stashenko *et al.* 1998, Barkhordar *et al.* 1999). The induction of Mn-SOD expression has been demonstrated following treatment with interleukin-1B, lipopolysacharide, tumour necrosis factor-α and also after tissue insult (Hassan 1988, Visner *et al.* 1990, Yoneda *et al.* 1992). These findings suggest that the inflamed dental pulp may release specific soluble factors that cause a higher increase in Mn-SOD transcription compared with Cu, Zn-SOD.

Conclusion

The development of pulpitis was associated with an elevated transcription of both Cu, Zn-SOD and Mn-SOD and appear to confirm the concept of the potentially different role of each SOD isoenzyme during pulp inflammation, however, this issue needs to be further clarified.

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