be tested to calibrate the examiners. However, most studies employing organoleptic measurements have not calibrated the examiners using such VSC mixtures or even H_2S or CH_3SH alone [51]. One consequence of this problem is that results cannot be compared between clinical studies, nor can different populations be compared.

Only VSCs, mainly produced at the tongue dorsum, correlate with oral malodor strength [52]. H_2S is a main component of physiologic halitosis, whereas CH_3SH is a main cause of oral pathologic halitosis involving periodontitis [52]. No other compounds have ever been shown to correlate with oral malodor [6, 18]. Instrumental methods for measuring VSC can be standardized, but reliable portable VSC detector with similar performance characteristics to a conventional GC has not been available. In this study we demonstrated that the OralChroma™ employing the modified protocol of Tangerman *et al* [27] and Twin Breasor™ can substitute adequately in the clinic for conventional GCs. Therefore, a GC, Twin Breasor™ and OralChroma™ with the modified protocol are recommended for determining VSC concentrations in mouth air.

The randomized crossover design reduces variability and thus adds to the statistical power; moreover, it enables different products to be tested on the same subjects. The design has the advantage that the same subjects are used to test each treatment, and the statistical analysis employed here was relatively simple as the intent was to compare change after treatment with baseline levels. If one desires that the treatments be compared statistically among themselves a more sophisticated analysis employing analysis of variance and using repeated measurements could be used [53]. In the present study our aim was to assess the newly recommended protocol to examine the effect of a zinc compound that has already been reported to be very effective in reducing VSCs in mouth air [1, 2, 9, 39, 41], and not to compare various compounds; therefore, a placebo control was not necessary. Nevertheless it was evident from the data that treatment with both mouthwash and tongue paste combined or with mouthwash alone reduced the total VSC concentration to half that of the baseline at 3 h after treatment, whereas tongue-paste treatment alone had lost its efficacy in suppressing VSC production at the 3 h time period. $ZnCl_2$-containing spray showed the weakest effect in reducing VSCs among the products tested. Thus, this study employing a short-term protocol demonstrates that a number of means of applying $ZnCl_2$ can be effective. Further studies are required to determine the optimal procedures or combinations of procedures to use this effective oral-malodor suppressing agent.

In conclusion we recommend the following standard for clinical research studies of halitosis: (a) they should be short term, typically 3 h in duration. (b) A randomized crossover study should be employed, as this reduces variability and thus adds to statistical power and also enables different products to be tested on the same subjects. (c) Measurements of oral malodor should be carried out preferably by using a GC, but a portable GC such as OralChroma™ or Twin Breasor™ may be acceptable after compensating for the effect of other volatiles appearing in the chromatograms.

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The role of p53 in an apoptotic process caused by an oral malodorous compound in periodontal tissues: a review

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Abstract

Oral malodor is caused by volatile sulfur compounds (VSCs) composed mainly of hydrogen sulfide (H_2S) and methyl mercaptan. In particular, H_2S is an important compound, since it is a major component of physiologic halitosis. The toxicity of VSCs is similar to that of hydrogen cyanide, and is well investigated. The role of VSCs in reducing collagen in human gingival fibroblasts is one of the main sources of their toxicity to human oral tissues. It has been reported recently that H_2S may cause apoptosis in several periodontal tissues. In human gingival fibroblasts, H_2S inhibits not only cytochrome c oxidase activity but also superoxide dismutase activity. The levels of reactive oxygen species are markedly increased, which causes the release of cytochrome c into the cytoplasm, resulting in caspase-9 activation; finally, the executor caspase, caspase-3, is activated. This pathway is commonly observed in cells from all periodontal tissues. Moreover, p53, an apoptotic factor, and phosphorlylated p53, which is the activated form, are increased by H_2S in keratinocyte stem cells and osteoblasts. H_2S also increases the expression of Bax, a primary response gene playing an important role in p53-mediated apoptosis, but maintains a lower expression of Bcl-2, an anti-apoptotic factor, in osteoblasts. It is concluded that the Bax apoptotic pathway and the mitochondrial pathway are activated by H_2S.

Introduction

Oral malodor is caused by volatile sulfur compounds (VSCs), mainly hydrogen sulfide (H_2S) and methyl mercaptan [1, 2]. Indeed, H_2S is always present in both physiological and oral pathological halitosis, and is a major component of physiological halitosis [3–5]. The toxicity of H_2S is similar to that of hydrogen cyanide; H_2S is recognized as a potent inhibitor of cytochrome-c oxidase (COX), a key enzyme of the respiratory chain in the mitochondria that produces adenosine triphosphate (ATP) [6]. COX inhibition by H_2S has been observed in some tissues and in purified COX [6, 7]. The key mechanism by which H_2S inhibits COX is in its binding to the heme iron of the enzyme, thus completely inhibiting the aerobic metabolism producing ATP [7].

Therefore, some studies have focused not only on the esthetic problems of VSCs but also on their toxicity to oral tissues, especially periodontal tissues. Studies have shown that increased levels of H_2S in the oral environment demonstrate highly toxic effects on periodontal tissues and play a role in the etiology and development of periodontitis [8–11]. Moreover, the activation of osteoclasts *in vitro* and in rat alveolar bone [10, 11] has been reported.

The apoptotic process is also involved in periodontal pathology; apoptosis plays an important role in the onset and progress of periodontal conditions [12–17]. A disruption of this between cell loss and cell production might induce periodontal conditions. The two main mechanisms in the apoptotic process are an intrinsic pathway involving the

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mitochondria and an extrinsic pathway involving a receptor–ligand-mediated mechanism [16, 17].

Recently, it has been shown that H_2S induces apoptosis in cells from several human periodontal tissues. Apoptosis in human periodontal tissues may be able to develop or control inflammation as well as cell destruction in periodontitis [18, 19]. If apoptosis of the cells is delayed, inflammatory cells may stay longer in periodontal tissues. Furthermore, excessive cytokine secretion following the process described above may cause periodontal destruction [20–22].

p53, a Bcl-2 family member, is one of the triggers of apoptosis in periodontal tissues [23]. p53, a typical tumor suppressor gene, functions as a transcriptional activator. Apoptosis, DNA damage and its repair are controlled by p53 [24]. p53 can also initiate cell-cycle control and avert further degradation of genomic DNA; p53 prevents replication of damaged DNA by apoptosis [25]. Since H_2S strongly inhibits superoxide dismutase, and as huge amounts of superoxide species are produced, it is clear that the mitochondrial membrane becomes depolarized and thus the intrinsic pathway is activated [23]. In the extrinsic pathway, caspase-8 is activated by a death-inducing signal complex with specific membrane receptors, and also by a pathway requiring p53 [14]. It has been shown that specific phosphorylation of p53 regulates biological activities such as growth arrest or apoptosis [26]. Phosphorylation of serine-46 is involved in the p53-dependent apoptotic process after genomic DNA damage. In periodontal tissues, it has been shown that the main pathway causing apoptosis is the intrinsic pathway which may involve p53 [23]. Thus, p53 might play an important role in the process. In this paper, we refer to the papers reporting apoptosis in periodontal tissue cells caused by H_2S in headspace air, and the current discussion is also focused on the role of p53 in the apoptosis of periodontal tissues.

Apoptosis of gingival fibroblasts

After fibroblasts are incubated with H_2S in headspace air, severe DNA fragmentation is found and the percentage of apoptotic cells increases in a time-dependent manner (figures 1 and 2) [27, 28]. H_2S inhibits COX, resulting in increased levels of reactive oxygen species (ROS) in the mitochondria, and collapses the mitochondrial membrane potential; the increased production of ROS in mitochondria causes disruption of the electrochemical gradient across the inner mitochondrial membrane [9]. H_2S at the lowest concentration in periodontal pockets or relatively healthy gingival crevices strongly inhibits both CuZn-SOD (superoxide dismutase) and Mn-SOD. Moreover, the percentage inhibition of hepatocyte growth factor (HGF)-SOD by H_2S was 79.6 ± 5.1 and a significantly increased amount of ROS was measured [27], indicating that an increase in the amount of ROS may be induced by SOD inhibition as well as COX inhibition caused by H_2S. Furthermore, it has been suggested that the increased ROS caused by SOD inhibition with H_2S might be carcinogenic (figures 3 and 4).

Increased ROS causes a significant loss of the mitochondrial inner transmembrane potential as described

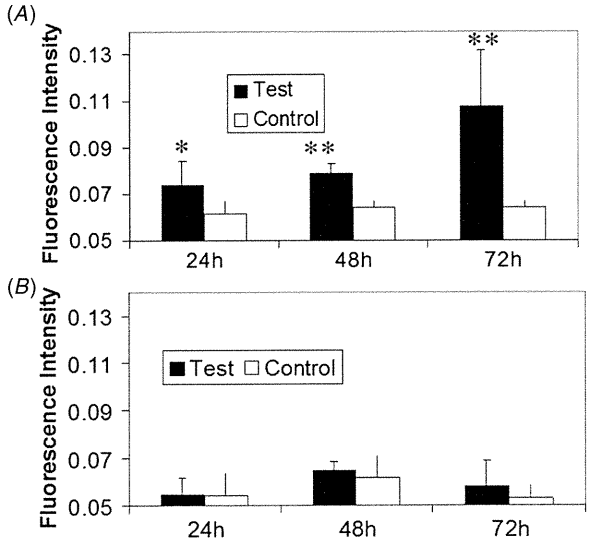

Figure 1. DNA fragmentation and hydrogen sulfide incubation time in human gingival fibroblasts. DNA fragmentation was assayed using the cell death detection ELISA kit^{TM}. (*A*) DNA fragmentation in the cells. (*B*) DNA fragmentation in the medium ($^*p < 0.05$, ANOVA). Reused under license from [27].

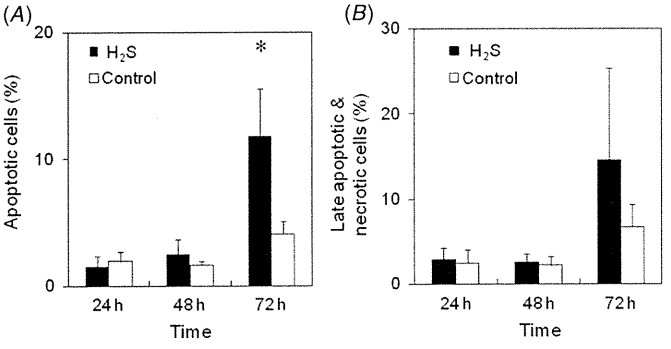

Figure 2. Comparison of apoptotic to necrotic and late-apoptotic cells induced by H_2S in human gingival fibroblasts. Apoptosis levels were detected by flow cytometry. (*A*) Percentage of early apoptotic cells. After 72 h, a significant difference was found between samples and the corresponding control. (*B*) Late apoptotic and necrotic cells. After 72 h, the levels of necrosis and late apoptosis were greatly increased. Each bar represents the mean ± SD of five independent experiments ($^*p < 0.05$, ANOVA). Reused under license from [27].

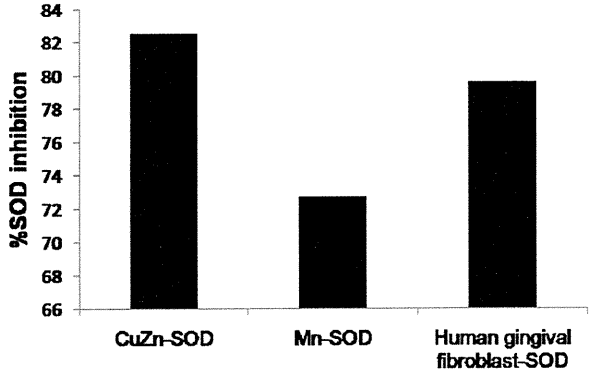

Figure 3. SOD inhibition in human gingival fibroblasts. %SOD inhibition was determined by the SOD Assay Kit-WST. Reused under license from [27].

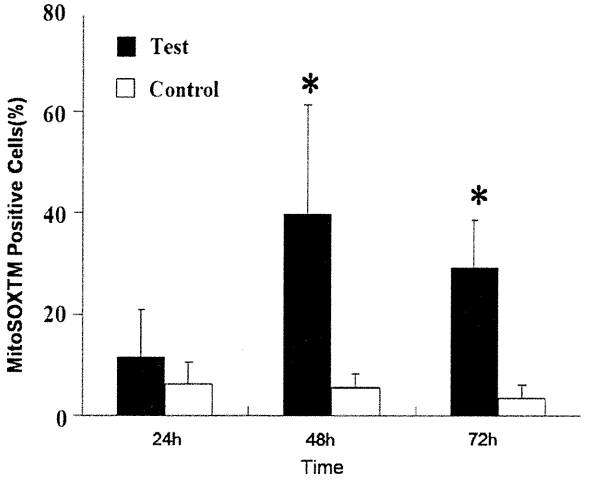

Figure 4. ROS produced by H_2S, and MitoSOX positive cells producing ROS. After H_2S incubation, a significant difference was found among the test groups. Each bar represents the mean ± SD of five independent experiments ($^*p < 0.01$, Mann–Whitney test). Reused under license from [27].

Table 1. Caspase activity in human gingival fibroblasts. Reused under license from [28].

	24 h	48 h		
	Caspase-9	Caspase-3	Caspase-9	Caspase-3
Control	0.2 ± 0.1	0.22 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Sample	2.9 ± 1.3[a]	2.9 ± 1.3[a]	4.1 ± 3.2[a]	4.1 ± 3.2[a]

Each bar represents the mean ± SD of five independent experiments ([a]$p < 0.05$, ANOVA).

above. This is followed by the release of cytochrome c from the mitochondrial inner membrane into the cytosol. Cytochrome c activates caspase-9, an upstream caspase, which in turn activates caspase-3. In HGF, caspase-9 was activated, followed by caspase-3 (table 1) [28]. Caspase-3 activation ultimately leads to DNA damage and apoptosis. The inactivity of caspase-8 suggests that the extrinsic pathway is not involved in this process.

Gingival epithelial tissues, especially the gingival crevicular epithelial tissue, play an important function in periodontal pathogenesis by producing a barrier against infiltration by periodontal pathogens and the destructive products of micro-organisms [29]. It is known that VSCs increase the permeability of the gingival crevicular epithelial model [29]. Other toxic effects of VSCs to HGF are reported to include the enhancement of the secretion of prostaglandin, cAMP and procollagenase [30]. VSCs increase collagen degradation and reduce collagen synthesis in HGF [31, 32], and increased levels of VSCs also delay the wound healing process *in vivo,* thus playing an important role in periodontal pathogenesis [33].

Apoptosis of gingival epithelial cells

VSCs also inhibit the proliferation of human gingival epithelial cells (HGECs) [34]. Among gingival tissues, epithelium plays a key function as a barrier against pathogens or toxic

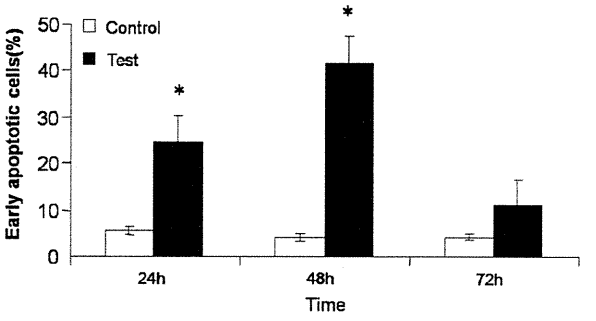

Figure 5. Hydrogen sulfide triggers apoptosis in epithelial cells derived from human gingiva. Apoptosis levels were detected by flow cytometry. (*A*) Percentage of early apoptotic cells. After 24 and 48 h, a significant difference was found between samples and the corresponding control. (*B*) Late apoptotic and necrotic cells. After 72 h, the levels of necrosis and late apoptosis were greatly increased. Each bar represents the mean ± SD of five independent experiments ($^*p < 0.05$, ANOVA). Reused under license from [35].

compounds as described above. The conservation of the epithelial barrier is therefore essential for maintaining the normal barrier function. During the progression of periodontal disease, this barrier can be affected.

In HGECs, both the levels of ROS, depolarization of the mitochondrial membrane and early apoptosis, were increased by H_2S exposure (table 2, figure 5) [35]. Caspase-9 levels were also markedly increased. Finally, H_2S exposure increased the caspase-3 activity, an executioner caspase, leading to the final stage of the apoptotic process (table 3).

However, caspase-8 activity stayed low. Increased casepase-8 activity initiates receptor–ligand-mediated apoptosis. The low activity of the enzyme indicates that the extrinsic pathway, receptor–ligand-mediated apoptosis, is not involved in H_2S-induced apoptosis in gingival epithelial cells (table 3) [35]. These results suggest that H_2S induces apoptosis by activating the intrinsic pathway, mitochondria-mediated apoptosis, in HGEC (figure 5).

Genomic DNA damage was detected using single-cell gel electrophoresis (Comet Assay; Trevigen, Gaithersburg, MD, USA). DNA involves a highly organized structure which deteriorates when damaged. The procedure is very practical and involves a simple theory. Briefly, the cells lysed using a lysis solution are placed in an electrophoretic field. After electrophoresis, DNA in the cells is stained with SYBR green, and then image analysis of the damaged DNA is carried out using both the fluorescence microscope and imaging software. Image analysis is based on the principle that intact DNA migrates extremely slowly during electrophoresis compared to the damaged DNA, which moves much faster than intact DNA because of the small DNA fragments. In image analysis, the cell is shaped like a comet; the head of the comet corresponds to the intact nucleus DNA region and its tail to the fragments of damaged DNA. The number of DNA strand breaks is correlated with the DNA comet tail shape; the size or shape of the tail represents the level of DNA damage, i.e. the number of DNA strand breaks is correlated with comet-tail parameters, such as the tail length and percentage of DNA in the tail, and their product, the tail moment. Genomic DNA damage as well as apoptosis was found in HGECs after exposure to H_2S

Table 2. Mitochondrial changes in epithelial cells derived from human gingiva. Reused under license from [35].

	ROS(%)		Depolarization (%)		Cytochrome c (ng ml^{-1})	
	24 h	48 h	24 h	48 h	24 h	48 h
Control	7.6 ± 3.6	9.1 ± 3.7	14.5 ± 5.0	19.6 ± 5.5	0.02 ± 0.01	0.02 ± 0.01
Sample	56.6 ± 4.1[a]	56.6 ± 4.1[a]	45.8 ± 11[a]	38.1 ± 10.5[a]	0.12 ± 0.02[b]	0.21 ± 0.02[b]

Each data point represents SD of five independent experiments ([a]$p < 0.01$, [b]$p < 0.05$, ANOVA).

Table 3. Caspase activities in epithelial cells derived from human gingiva. Reused under license from [35].

	24 h			48 h		
	Caspase-9	Caspase-3	Caspase-8	Caspase-9	Caspase-3	Caspase-8
Control	5.3 ± 2.5	3.3 ± 1.1	4.5 ± 2.3	4.6 ± 1.1	3.0 ± 1.2	2.8 ± 2.1
Sample	18.7 ± 3.8[a]	25.6 ± 8.4[a]	5.5 ± 2.1	51.1 ± 2.8[a]	46.1 ± 8.0[a]	7.0 ± 1.5

Each bar represents the mean ± SD of five independent experiments ([a]$p < 0.05$, ANOVA).

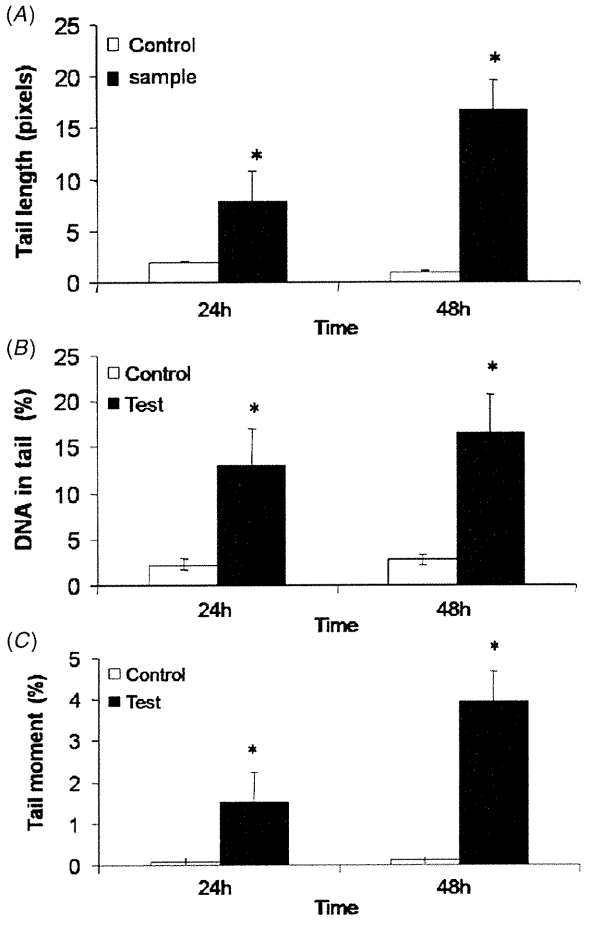

Figure 6. Genomic DNA damage in human gingival epithelial cells. Genomic DNA damage was detected using three different parameters. (*A*) Tail length, expressing distance of damaged DNA migration from the nucleoid, was significantly increased after 24 and 48 h compared with their controls. (*B*) Percentage of DNA in tail was increased after both 24 and 48 h. (*C*) Tail moment, representing the product of the first two parameters, was significantly increased at each time point compared with their controls. Each bar represents the mean ± SD of five independent experiments; 75 nuclei analyzed per experiment (*$p < 0.05$, ANOVA). Reused under license from [35].

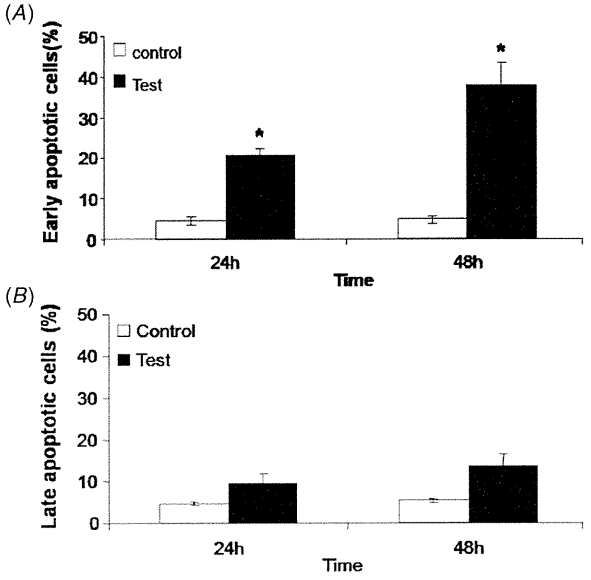

Figure 7. H_2S triggers apoptosis in keratinocyte stem cells. H_2S triggers apoptosis in keratinocyte stem cells. (*A*) Percentage of early apoptotic cells after both 24 and 48 h, a significant difference was found between samples and their corresponding controls. (*B*) Late apoptotic and necrotic cells were <10% at each time point. Each bar represents the mean ± SD of five independent experiments (*$p < 0.05$, ANOVA). Reproduced with permission from the American Academy of Periodontology [23].

(figures 6(*A*)–(*C*)) [35]. It is also suggested that the p53 pathway, which is initiated by DNA damage, might be involved in the apoptotic process and that H_2S may have pathological effects on human gingival epithelia at the genomic level [23].

Apoptosis of keratinocyte stem cells: the role of p53

Keratinocyte stem cells play a key role in maintaining gingival crevicular epithelium which composes the periodontal tissues against invasion of periodontal pathogens and their products. Thus, crevicular epithelium is the first barrier against bacterial infiltration. It has been found that H_2S induces apoptosis in these cells (figure 7). As can be seen in HGFs or HGECs, caspase-8 activity levels remained very low after H_2S exposure

Table 4. Caspase-3, 8, 9 activities in keratinocyte stem cells. Reused with permission from the American Academy of Periodontology [23].

	24 h			48 h		
	Caspase-9	Caspase-8	Caspase-3	Caspase-9	Caspase-8	Caspase-3
Control	0.05 ± 0.01	0.24 ± 0.05	0.04 ± 0.01	0.06 ± 0.02	0.26 ± 0.05	0.05 ± 0.02
Sample	3.08 ± 0.41[a]	0.17 ± 0.06	0.49 ± 0.13[a]	5.67 ± 0.42[a]	0.19 ± 0.08	0.76 ± 0.18[a]

Each bar represents the mean ± SD of five independent experiments ([a]$p < 0.05$, ANOVA).

Table 5. Genomic DNA damage in keratinocyte stem cells. Reused with permission from the American Academy of Periodontology [23].

	24 h			48 h		
	Tail Length (px)	%DNA (Percentage)	Tail Moment	Tail Length (px)	%DNA (Percentage)	Tail Moment
Control	2.17 ± 1.01	2.26 ± 0.05	0.09 ± 0.03	1.02 ± 0.09	2.72 ± 0.4	0.11 ± 0.05
Sample	7.82 ± 2.94[a]	12.92 ± 4.12[a]	1.51 ± 0.55[a]	16.71 ± 6.86[a]	16.52 ± 4.14[a]	3.95 ± 0.71[a]

Each data point represents means ± standard deviations of five independent experiments; 75 nuclei analyzed per experiment ([a]$p < 0.05$).

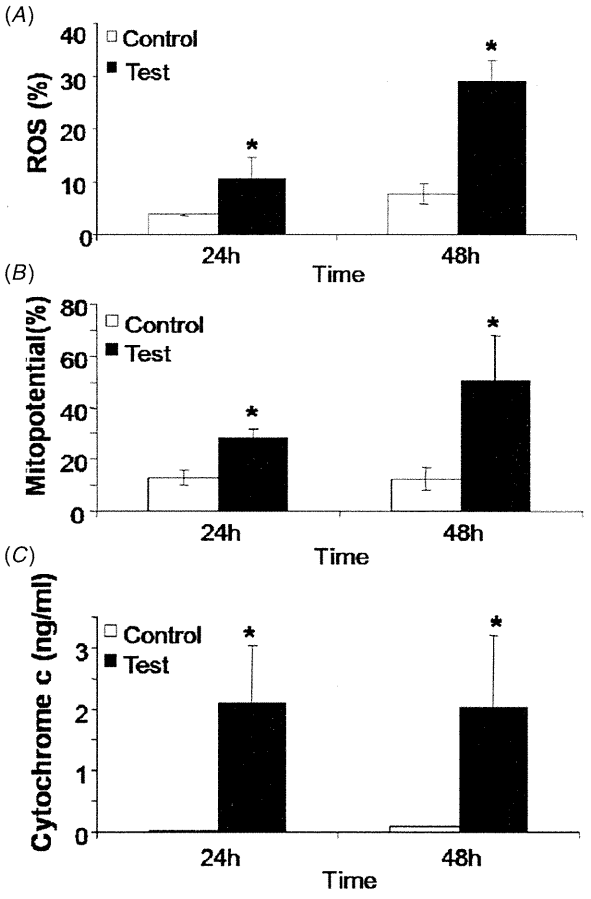

Figure 8. Mitochondrial changes in keratinocyte stem cells. (*A*) ROS was significantly increased compared to their controls. (*B*) Mitochondrial membrane electrical gradient was disrupted after 24 and 48 h. (*C*) Cytochrome c into cytosol was significantly increased compared to the corresponding control groups. Each bar represents the mean ± SD of five independent experiments (*$p < 0.05$, ANOVA). Reused with permission from the American Academy of Periodontology [23].

of keratinocyte stem cells, while caspase-9 and caspase-3 were increased in a time-dependent manner (table 4) [23]. DNA fragmentation parameters, such as the tail length, percentage of DNA in the tail and the tail moment, were also increased. As a result, DNA strand breaks were dramatically increased after H_2S exposure (table 5) [23].

After H_2S exposure ROS was significantly increased (figure 8(*A*)), and the number of mitochondrial membrane-depolarized cells was also increased (figure 8(*B*)). Consequently, a significant amount of cytochrome c was released into the cytosol (figure 8(*C*)).

Therefore, the extrinsic pathway is not involved in H_2S-induced apoptosis. Bax, a member of the Bcl-2 protein family, is a p53 primary response gene causing p53-mediated apoptosis. The Bcl-2 family is composed of two groups involving exactly opposite functions: one is an initiator of apoptosis like Bax and another suppresses apoptosis as Bcl-2 does. A tumor-suppressor gene, p53, can act as the main defense mechanism against cancer by activating apoptosis and exterminating the cancer cell. Both total and phosphorlylated p53 are increased by H_2S exposure (figure 9), and Bax also increases in a time-dependent manner (figure 10) [23]. Bax, in general an apoptosis-promotion gene, usually exists in the cytoplasm; it shifts to mitochondria as a response to the apoptosis-inducing signal. An increase in ROS levels collapses the mitochondrial transmembrane potential, which is a key process for the release of cytochrome c, and then activates caspase-9 and caspase-3. H_2S triggers apoptosis in keratinocyte stem cells by activating a p53-mediated apoptotic pathway, followed by the activation of the mitochondrial pathway as described above.

Apoptosis of osteoblasts: the role of p53

Alveolar bone loss is one of the most important changes in the development of periodontitis as well as the destruction of connective tissues. H_2S also induces apoptosis in osteoblasts. The total p53 level in H_2S-exposed cells was significantly increased compared to their respective controls (48.45 ± 4.76 versus 15.04 ± 1.44 pg ml^{-1} for 24 h; 70.99 ± 5.70 versus 12.57 ± 2.62 pg ml^{-1} for 36 h; and 72.50 ± 6.65 versus 15.51 ± 3.82 pg ml^{-1} for

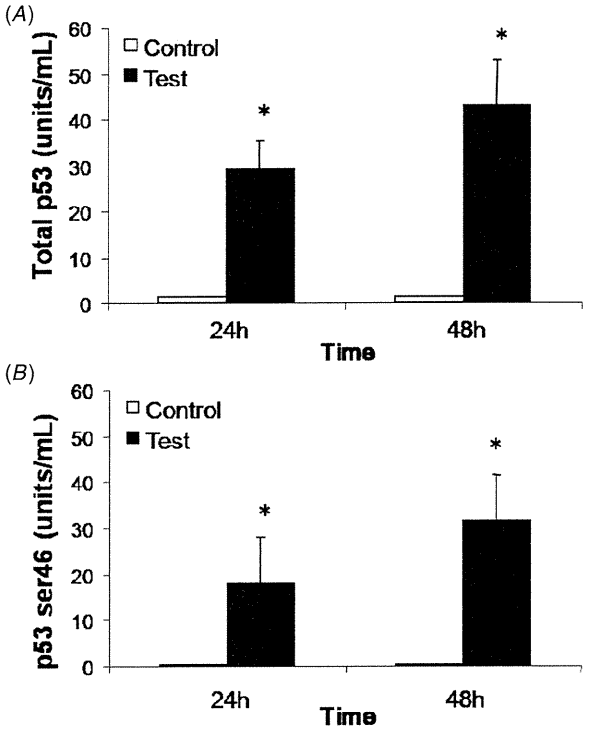

Figure 9. Effect of H_2S on p53 levels in keratinocyte stem cells. Total p53 (*A*) and p53 phosphorylated at serine 46 (*B*) were analyzed using enzyme-linked immunosorbent assay and were increased after 24 and 8 h of incubation. Each bar represents the mean ± SD of five independent experiments ($^*p < 0.05$, ANOVA). Reused with permission from the American Academy of Periodontology [23].

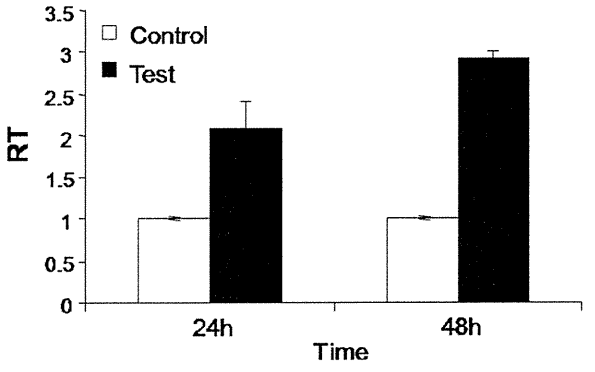

Figure 10. Effects of H_2S on Bax gene expression. Gene expression was measured using quantitative RT-PCR to compare cells treated with H_2S to their controls. Each bar represents the mean ± SD of five independent experiments ($^*p < 0.05$, ANOVA). Reused with permission from the American Academy of Periodontology [23].

48 h, for $p < 0.01$ and $n = 5$) [36]. p53 initiates the cell-death ligand/receptor pathway including caspase-8, one of the major pathways involved in apoptosis.

In the intrinsic pathway, p53-primary-response molecule is Bax [37]. Consequently, Bax causes mitochondrial membrane depolarization, which induces the release of cytochrome c from the mitochondria [23, 37].

After H_2S exposure, the Bax gene was upregulated in a time-dependent manner, whereas Bcl-2, an anti-apoptotic gene, stayed low. Following H_2S incubation, depolarization of the mitochondrial membrane was significantly increased at 24, 36 and 48 h, respectively [36]. Following membrane depolarization, the cytochrome-c level in the cytoplasm of H_2S-treated cells was significantly higher than in the controls at 24, 36 and 48 h, respectively. Cytochrome c in cytosol starts the apoptotic cascade, and activates the initiator caspase-9. Downstream, the cascade activates caspase-3, an executor enzyme. Also, a significant increase in the activity of both caspase-9 and caspase-3 was found after H_2S exposure [36]. The mitochondrial pathway was activated by H_2S exposure during the apoptotic process in osteoblasts as well as in other periodontal cells.

Both caspase-9 and caspase-8 are initiator caspases activating caspase-3, the executor of apoptosis. The receptor–ligand-mediated apoptosis mechanism was also examined; in this study, caspase-8 is an initiator caspase in the extrinsic pathway mentioned above. The activation of the caspase is associated with Fas, TNF-R1 and TRAILs [38–41]. Then, caspase-8 is activated with both inducible dimerization and inducible cleavage. It was inferred that caspase-8 was activated in a time-dependent manner following H_2S exposure; however, TRAILs never activate caspase-8 in osteoblasts [42, 43].

Therefore, caspase-8 activation in this study could not have been caused by TRAILs. Also, both Fas and Fas-ligand activity levels remained low, suggesting that they are not involved in H_2S-induced apoptosis. Many cytokines including TNF-α activate caspase-8 and start the apoptotic process [44–51]. Therefore, the activities of TNF-α and other several inflammatory cytokines were determined after H_2S exposure [52, 53]. There was no difference in TNF-α, IL-β, IL-2, IL-4, IL-10, IFN-γ, G-CSF and GM-CSF levels between the tests and their respective controls.

Consequently, the cell-death ligand and receptors in the extrinsic pathway may not be involved in osteoblasts apoptosis caused by H_2S, whereas caspase-8 is activated during the apoptotic process. Previous studies clearly demonstrated that p53 directly initiates caspase-8 independent of the extrinsic pathways [38–40]. Both p53 and caspase-8 were significantly activated after H_2S exposure, while no cell-death ligand and receptors were activated [36]. This result suggests that caspase-8 can be activated in the absence of a death-receptor signaling and caused by p53 as reported previously [38–40]. Caspase-8 activation by H_2S might be directly associated with p53.

Conclusion

In conclusion, H_2S at concentrations normally found in human gingival crevicular fluid induces apoptosis in gingival fibroblasts, gingival epithelial cells, keratinocyte stem cells and osteoblasts. H_2S triggers apoptosis by activating a p53-mediated apoptotic pathway in keratinocyte stem cells and osteoblasts, and it is suggested that H_2S may also activate p53 in other cells. Only in osteoblasts among these periodontal tissues do the molecular mechanisms underlie those apoptotic processes that include caspase-8 activation. In such a process, p53 activates both caspase-8 and caspase-9.

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Advances in breath odor research: re-evaluation and newly-arising sciences

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EDITORIAL

Advances in breath odor research: re-evaluation and newly-arising sciences

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The articles in this special section describe the most recent advances in halitosis research presented at the Ninth International Conference on Breath Odor Research, a joint conference with the XXIV CONBRAPE (Brazilian Congress of Periodontology) held at Bahia Othon Palace Hotel in Salvador, Bahia, Brazil on 25–28 May 2011. It has been almost half a century since Joseph Tonzetich of the Faculty of Dentistry at the University of British Columbia, Vancouver, Canada (the first honorable member of the International Society for Breath Odor Research (ISBOR) who died in 2000, and is known as 'the godfather of halitosis research') published his first halitosis paper entitled 'Evaluation of volatile odoriferous components of saliva' in *Archives of Oral Biology* in 1964 [1]. This was the starting point for breath-odor research, long before ISBOR was established, although research in this area had declined by the time we convened global collaboration in halitosis research.

One must ask the question; what progress have we made during the past half-century? Although a few bad-breath detectors have appeared on the market, organoleptic scoring is still likely to remain as the 'gold standard'. In the 21st century, people in modern dentistry or medicine are still sniffing their patients' breath in order to diagnose halitosis, a very subjective method! Halitosis is widely believed to be simple to diagnose and treat, but this is certainly not the case, considering we have not yet even completed an objective detection measurement protocol for oral malodor.

In the treatment of halitosis, for many years before Tonzetich came into the field, tongue coating had been suspected to be the main cause, and this was scientifically proven a quarter of a century ago. However, people still visually and subjectively measure the amount of tongue coating to diagnose halitosis and an objective and more accurate method is required and needs to be developed. Those of us that work in this field employ mainly subjective protocols for diagnosis, but scientists are well aware that the data obtained by subjective procedures are not universal and therefore it is not possible to compare data obtained by a subjective measure with data from a different paper. It has been suggested that our procedures in halitosis clinics are not scientific and are almost the same as they were hundreds of years ago. Why have we not made progress in these fundamental measures for halitosis clinics? Everybody involved in halitosis clinics or the related sciences should ask themselves this question.

Removing tongue coating (TC), which consists mostly of bacteria and exfoliated keratinized epithelial cells, is a radical remedy and is considered as the most effective way to treat halitosis. For many years, people employed mechanical tongue-cleaning methods, such as using a tongue scraper. In the early 1970s it was reported that mechanical stimulation promotes tongue cancer [2] . This hypothesis may not be absolutely correct, since most tongue cancers are found on the side (lateral margin) of middle third of the tongue, and so the relationship between tongue scraping and cancer has not yet been confirmed. However, there is still a possibility that mechanical stimulation is one of the causes of tongue cancer. To effectively and safely remove tongue coating there is no doubt that we must develop a novel technology without using mechanical

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stimulation, but no such research efforts have yet been reported since the early 1970s.

The protocols for halitosis clinical research can be divided into two sub-classifications: short-term and long-term. For both groups, subjects must abstain from ingestion of food, drinking and oral hygiene for four hours prior to evaluation of oral malodor, as described in [3], since this is the best time to measure volatile sulfur compounds (VSCs), as defined by the ADA guidelines. A recent paper has suggested that this protocol might not be correct, since eating and oral hygiene may affect VSCs in mouth air for longer than four hours after these activities [4]. In long-term studies, three weeks' intervention is recommended by the ADA, during which time they measure malodor strength or VSC concentration at the baseline each day. The premise of this protocol is that the baseline of VSC concentration or malodor strength is constant over certain days, but there are those who doubt that the baseline is consistent.

The organoleptic procedure is perceived as the gold standard for diagnosing oral malodor; however, for clinical work on halitosis, not only detection but also quantification is required. Organoleptic measurements do not require purpose-build apparatus, which explains why this has been a popular method among clinicians. But there are many drawbacks to this type of diagnosis. Quantification of odor sensations is very difficult, and the most difficult feature of the organoleptic procedures is stimulus presentation, while objective measurements directly determine the concentration of stimulus. Therefore, halitosis detectors should be the method of choice for clinicians.

A number of halitosis detectors have been used in the past 30 years. The portable sulfide monitor was very popular, but it also reacts with other compounds which cannot be accurately detected. Recently, portable gas chromatographs (GCs) were introduced, but this exciting technology could only report a few evaluations. Even using portable or regular GCs, the ADA recommends that their protocols be employed in clinical research.

The papers selected for this special section provide some answers to the above questions or demonstrate a novel aspect of halitosis pathology. Moreover, they illustrate the diversity of approaches to the pathological and physiological activities of VSCs. It is a challenging field.

The main challenges, as noted by K Yeagaki *et al* [5], are that problems have been identified in current, widely-used protocols. (1) The baselines of VSC concentrations in mouth air varied considerably over the course of a week. (2) When subjects refrained from eating, drinking and oral hygiene, including mouth rinsing, the VSC concentrations remained constant until the subject began eating again. (3) Over a six-hour period after a meal and oral hygiene, VSC concentrations decreased significantly. The above data point to optimal times and conditions for sampling subjects. Yeagaki *et al* also compared measurements obtained using several portable devices with measurements obtained using GCs, showing that portable devices demonstrate capabilities similar to those of GCs. Thus, a recommended protocol has been established. The proposed protocol includes the following recommendations: (a) a short-term rather than long-term study is strongly recommended, since VSC concentrations are constant in the short term; (b) a crossover study would best avoid the effects of individual specificities on each clinical intervention; and (c) measurements of VSCs should preferably be carried out using either a GC or a portable GC.

To control VSC production, removing the TC is essential for the maintenance of oral hygiene and tongue-brushing or a scraper is utilized for this purpose. Mechanical stimulation needs to be eliminated as much as possible for the reasons mentioned above or to avoid unpleasant side effects. Nohno *et al* [6] have developed candy tablets containing a protease, actinidine, and effect of long-term use of these on both TC accumulation and the concentration of VSCs in mouth air has been determined. This is a novel procedure that removes the TC safely.

Although most researchers and clinicians evaluate TC accumulation subjectively and visually, this is neither scientific nor objective. Nohno *et al* have also demonstrated an objective method of evaluating TC using a digital camera and the software ImageJ (NIH, USA). Moreover, they suggest that a long-term research design might not be appropriate. To develop new halitosis sciences, ISBOR would never to nip new knowledge in the bud—we should nurture such a promising young bud.

Tangerman *et al* [7] found no association between halitosis and *H. pylori* infection of the stomach, although *H. pylori* is believed to be a cause of extra-oral pathologic halitosis. Suzuki *et al* [8] found that the use of probiotics, a now popular way for controlling oral malodor, is effective in reducing TC accumulation, but they also described its limitations. Determining the limitations of a remedy is essential for both clinicians and patients. We must encourage the adoption of such a presentation style as demonstrated by Suzuki *et al* among breath-odor clinical scientists or clinicians.

Possible chemosensory dysfunction can elicit halitosis complaints. Falcão *et al* [9] found that chemosensory dysfunction may cause pseudo-halitosis that proves very difficult to treat.

Aoyama *et al* [10] reviewed the role of p53 in the apoptosis of periodontal tissues caused by oral malodorous compounds, and emphasized its toxicity. This information could be very useful in enabling people to prevent halitosis. Ishkitiev *et al* [11] established the protocol for differentiation of human dental pulp stem cells to hepatic cells, and found that hydrogen sulfide increases hepatic differentiation. This is a positive effect of oral malodorous compounds. However, they still need to establish universally accepted standards for evaluating the toxicity or positive effects of VSCs as shown in their report, since those topics are not yet well understood among halitosis scientists.

I would like to emphasise that this editorial represents only my own views on some of the most interesting findings in halitosis research. However, I must stress again that it is time to re-evaluate the halitosis clinical sciences, and to invest in the newly-arising sciences of halitosis. We look forward to our next ISBOR conference in two years' time.

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Preventing Aspiration Pneumonia by Oral Health Care

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Abstract

In recent years, the need for oral care in preventing aspiration pneumonia has been recognized across the academic disciplines. Pursuing both oral cleaning and improvements in food ingestion is necessary to achieve the objective. At a minimum, cleaning should include mechanical cleaning of the tongue and palatal mucus and removal of pathogenic bacteria causing aspiration pneumonia (typically oral anaerobic bacteria). As for the improvement of eating function, the patient should be first trained in breathing and swallowing saliva so that he/she gradually regains swallowing function. Even if the patient has a gastric fistula, successful oral ingestion of several mouthfuls not only prevents aspiration pneumonia, but can also raise the quality of the social life by enhancing the patient and the family's motivation for everyday living.

Regardless of the patient's clinical condition, the oral cavity becomes moist and healthy in color as soon as it is cared for—and its care is reflected in the entire body. I believe that the oral cavity deserves greater recognition, and hope less people will have to suffer from ingestion problems and aspiration pneumonia through proper oral care in the future.

Key words Aspiration pneumonia, Eating function training, Oral care, Oral intake, Oral moisture retention in the oral cavity

Introduction

Pneumonia is the fourth most common cause of death among Japanese, and is the leading cause among the elderly in need of long-term care. Moreover, 30% of those who die of pneumonia are diagnosed with aspiration pneumonia.[1] Thanks to intervention studies and research, it is now recognized across the academic disciplines that regular oral care by a professional can prevent aspiration pneumonia and reduce the onset of fever.[2] However, even oral microorganisms that are not ordinary pathogenic tend to develop into infectious diseases as a result of opportunistic infections. Culture tests of bacteria obtained from patients who contracted aspiration pneumonia using the percutaneous pneumocentesis revealed that oral resident bacteria were most common.[3] Moreover, oral care not only prevents pneumonia, but has also been reported to have a positive impact on activities in daily life and cognitive functions.[4] Currently, the concept of oral care is broadly interpreted as *oral health care*, including not only oral cleaning but also eating function training. This paper will examine the characteristics of the oral cavities of elderly people in need of long-term care who developed aspiration pneumonia and the concept and techniques for oral care.

Characteristics of Oral Cavity Which Causes Onset of Aspiration Pneumonia

The oral cavities of patients who contract aspiration pneumonia have characteristic mucous membranes, soft tissue, teeth, and oral func-

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Fig. 1 Mucous membranes residue, remaining like an oblate

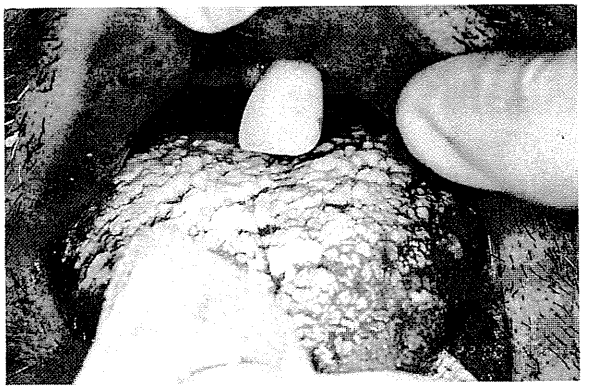

Fig. 2 Coated tongue

tion. The key points to observe to prevent aspiration pneumonia or improve the prognosis are described below.

Residue in oral mucous epithelium and oral dryness

When oral functions are almost completely suppressed due to depressed consciousness or tube feeding such as intravenous drips and gastric fistula, the secretion of mucosal resting saliva begins to dominate, mixing with the residue in the oral epithelium to form a sticky paste that adheres to the oral cavity membranes and teeth. Since the self-cleaning function of the oral cavity is not working, the oral mucous membrane is not regenerated and replaced. Instead, the mucous remains on the palate like an oblate as shown in **Fig. 1**, and the tongue becomes coated (**Fig. 2**). The bacterial flora of such palate or tongue contains different bacteria that do not normally appear in the healthy mouth, raising the risk of upper respiratory tract infections and thus aspiration pneumonia.

The main causative microorganism of aspiration pneumonia is thought to be gram-negative anaerobic bacteria, which live in secreta (phlegm), epithelial residue, crusts, and saliva. A silent aspiration of these microorganisms (called micro-aspiration) in the process of normal respiration causes pneumonia.

Morphological damage to oral hard tissue (teeth)

Inadequate oral cleaning can result in multiple and simultaneous cases of dental caries at the tooth cervix. If oral hygiene continues to be neglected, the crown can rot away with only residual roots left as shown in **Fig. 3**. Food residue tends to adhere to the residual root of the decayed teeth, creating bacterial plaque, and forming bacterial flora that cannot be removed by mere gargling. If the condition reaches the point that bad breath pervades the entire room, it is evident that decaying matter is remaining in the mouth, meaning there is a grave risk of aspiration pneumonia.

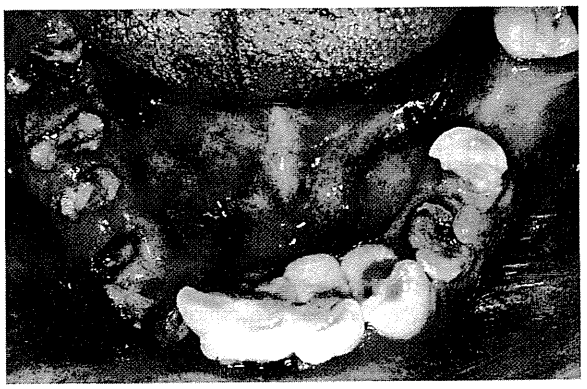

Fig. 3 Remaining roots after losing the crown portion

Impaired oral function

When using a cerebral stroke as an example, more than 50% of patients in the acute phase have aspiration, but only 7% still experience aspiration during the recovery phase. But because their oral cavity organs such as the lips, tongue, cheek, and soft palate remain paralyzed even during the recovery phase, 30% of chronic patients experi-

Fig. 4 Moisturizing agents

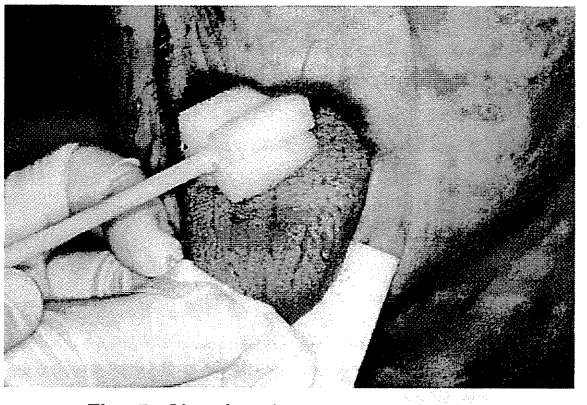

Fig. 5 Cleaning the tongue and palate

ence impaired mastication.

As the patient recover from acute phase and move on to chronic phase, impairment of eating functions shifts from the pharyngeal phase to oral phase.[5] Even if the patient succeeds in ingesting food orally, poor mastication prevents the formation of alimentary bolus, and food adheres to the surface of the teeth in its original shape. This kind of functional impairment of the oral phase is one cause of poor oral hygiene and consequently induces silent aspiration or misswallowing.

Approaches and Techniques for Oral Care

Aspiration pneumonia can be broadly divided into 2 groups: one caused by *micro-aspiration* (the aspiration of microorganisms into the trachea) and *macro-aspiration* (the aspiration of food). Micro-aspiration can be prevented by cleaning of the oral cavity. Macro-aspiration can be addressed by improving eating and swallowing functions. When pursuing both oral hygiene management and eating function restoration, the original objective of preventing aspiration pneumonia can be achieved.

Oral hygiene management

Moisture retention

The first step is to retain moisture in the oral cavity. There are 3 types of moisturizing agents: gels, creams, and liquids (**Fig. 4**). One may use a piece of gauze or cotton wrapped around the finger as a cleaning tool, but it is better to use an ordinary sponge brush or special brush specifically designed for the mucous membranes in order to more scrupulously and reliably enhance the effects of moisture retention and cleaning. After spreading a moisturizing agent to a brush, apply it to the upper and lower lips, left and right buccal mucous membranes, palate, and tongue, to care for the mucous membranes.

Moisturizing agents in gel form include a polymer (poly glyceryl methacrylate), which achieves a swelling effect by absorbing moisture and softening the mucous membranes.

Moisturizing creams include substances that promote a natural healing for oral inflammation with a combination of milk protein extracts containing antimicrobial ingredients and immunoglobulin. In particular, using a toothbrush to remove epithelial residue (shown in **Fig. 1**) or crusts can have an adverse result of bleeding. However, applying a moisturizing agent to the dry lining in advance softens the epithelium within a few minutes, making removal easier.

Some liquid moisturizing agents contain hyaluronic acid. Once the oral cleaning is finished, spraying the liquid moisturizer and then applying a moisturizing gel can extend the moisturizing effect for a longer period.

Areas that must be cleaned

In the oral cavity of a tube-fed patient, microorganisms are most often stuck to 2 areas: the tongue and palate. This means that even just mechanically cleaning the tongue and palate can help to prevent aspiration pneumonia (**Fig. 5**).

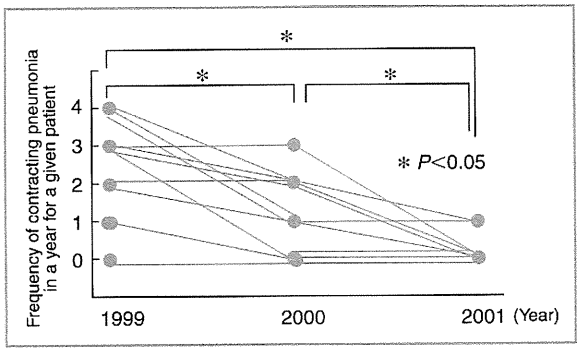

Fig. 6 Frequency of pneumonia in a 3-year period in the group that received both professional oral cleaning and swallowing training (11 patients)

Regarding eating function training

Key points for eating function

Mechanical stimulation from oral cleaning can stimulate the secretion of saliva and activate the swallowing and coughing reflexes. Thus, oral cleaning can be a very important anti-infection measure. When cleaning the mouth, attention should be paid to the way in which the patient disposes of saliva. In other words, if the visual observation confirms the closing of the lips and elevating of the larynx when the patient is swallowing saliva, no residual sound is noted in the pharyngeal region with a stethoscope, and the patient can expectorate when choking, then, the patient can proceed to aggressive swallowing training. Tests that utilize mechanical devices, such as videofluorography (VF) or video endoscopy (VE), should also be used for diagnosis as necessary.

As long as the patient is unable to control his/her breathing, mastication and deglutition movements will be difficult. So, first the swallowing training should start with the practice in abdominal breathing on the bed. Next, the patient can start training in swallowing saliva by licking candies to stimulate saliva secretion and induce saliva swallowing.

"A spoon of life"

In this section, I will discuss a comparative case study I conducted with my colleges.[6] At an intensive care home for the elderly, we provided oral care to patients with dementia once a week since 1999. Of these patients, a dental professional provided weekly oral cleaning to 10 dysphagia patients with feeding tube who needed assistance for all of their daily life activities. The outbreak frequency of pneumonia among these 10 patients was tracked over a period of 3 years. After 3 years, there was an overall decline in the frequency of pneumonia, but since there were several patients who experienced pneumonia repeatedly, the difference was not statistically significant in these 3-year period data.

However, at another facility, we provided functional training of dysphagia in addition to oral cleaning to 11 patients with similar physical conditions. The functional training was the ingestion of less than 40 ml of gelatin jelly once a week. Endoscopic diagnosis, visual examination, auscultation, and palpation were used to check for aspiration, and the patient only orally ingested the jelly when the dentist visited. Two of the 11 patients died of heart failure during the 3-year period. But as illustrated in **Fig. 6**, the remaining 9 patients showed a decrease in the frequency of pneumonia or no change, which was statistically significant.

In addition to daily oral cleaning by care givers, by providing weekly professional oral cleaning and the ingestion of a spoonful of jelly once a week, this study succeeded in reducing the frequency of pneumonia.[6] Even though it was only a mouthful, this ingestion of jelly was "a spoon of life." In the latter facility, more than 15 patients a year died of pneumonia prior to 1999, but the pneumonia mortality has decreased to less than 6 since 2001. These figures confirmed the importance of using eating function in patient care, even to ingest just a spoonful of gelatin jelly, in addition to proper cleaning.

Conclusion

The oral problems among elderly patients in need of long-term care become progressively worse as the period of systemic illness prolongs, to the point of disintegration of the crown, stagnation of food residue, adhesion of epithelial residue, and loss of artificial teeth. Before reaching such dismal conditions, at some point in the transition—from the acute to recovery phase or from the recovery to maintenance phase, there must have been a turning point somewhere.

In recent years, the need for oral care in preventing aspiration pneumonia has been recognized across the academic disciplines. In order to successfully prevent aspiration pneumonia, a

patient must: 1) improve his/her ability to ingest food items, and 2) receive sufficient oral care including proper cleaning. To this end, oral cleaning should at least include mechanical cleaning of the tongue and palate to remove pathogenic bacteria (e.g., anaerobic bacteria) that can lead to aspiration pneumonia. As for improving eating function, the patient should first be trained in breathing and swallowing saliva in order to gradually regain swallowing function. Ingestion of several mouthfuls can successfully prevent aspiration pneumonia, even if the patient is being tube fed. The act of eating, even as little as a spoonful, can also raise motivation for life for both the patient and the family and can enhance the quality of their social life.

The oral cavity is an organ that responds promptly to proper care, regardless of the clinical condition. It becomes moist and healthy in color as soon as it is cared for, and the improved oral cavity can also influence the entire body. "Eating" is such a simple, everyday activity for healthy people, and so is oral care. But for those who have problem with eating function, "it is only oral care, but it is part of the care." I would like to see greater recognition of oral care, so that less patients and people in recuperation will have to suffer from ingestion problems and aspiration pneumonia.

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