Original Article

Protective effects of (6R)-5,6,7,8-tetrahydro-L-biopterin on local ischemia/reperfusion-induced suppression of reactive hyperemia in rat gingiva

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We herein investigated the regulatory mechanism in the circulation responsible for rat gingival reactive hyperemia (RH) associated with ischemia/reperfusion (I/R). RH was analyzed using a laser Doppler flowmeter. RH and I/R were elicited by gingival compression and release with a laser Doppler probe. RH increased in a time-dependent manner when the duration of compression was between 30 s and 20 min. This increase was significantly suppressed by N^{ω}-nitro-L-arginine-methyl-ester (L-NAME), 7-nitroindazole (7-NI), and 2,4-diamino-6-hydroxypyrimidine (DAHP). However, RH was markedly inhibited following 60 min of compression. This inhibition was significantly decreased by treatments with superoxide dismutase (SOD), (6R)-5,6,7,8-tetrahydro-L-biopterin (BH$_4$), and sepiapterin. The luminescent intensity of superoxide anion (O$_2$$^{\bullet-}$)-induced 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a]pyrazine-3-one (MCLA) was markedly decreased by SOD and BH$_4$, but only slightly by sepiapterin. BH$_4$ significantly decreased O$_2$$^{\bullet-}$ scavenging activity in a time-dependent manner. These results suggested that nitric oxide (NO) secreted by the nitrergic nerve played a role in regulating local circulation in rat gingiva. This NO-related regulation of local circulation was temporarily inhibited in the gingiva by the I/R treatment. The decrease observed in the production of NO, which was caused by suppression of NO synthase (NOS) activity subsequent to depletion of the NOS co-factor BH$_4$ by O$_2$$^{\bullet-}$, played a partial role in this inhibition.

Key Words: nitric oxide synthases, reperfusion, BH$_4$, gingiva, reactive hyperemia

S ince blood supply, which includes oxygen, nutrients, and immunocytes, is essential for the homeostatic maintenance of tissues, regulatory mechanisms in the local circulation play important roles in homeostasis. Increases in postischemic-reperfusion blood flow, that is, reactive hyperemia (RH), represent an efficient vasodilative response of the blood supply to ischemic tissues following the temporary occlusion of blood supply vessels, i.e., a compensatory circulation reaction. Humoral, myogenic, and neurogenic factors have been suggested to play roles in vasodilative regulation in RH.[1]

We previously demonstrated that nitric oxide (NO) was a mediator of postcompression-reperfusion RH in dog gingiva.[2,3] A relationship between NO and RH has been reported in tissues and organs other than those in the oral cavity region (for example, the extremities, heart, and brain).[4–6] NO is produced from L-arginine as a substrate by nitric oxide synthase (NOS). The physiological actions of NO are not limited to vascular smooth muscle relaxation,[7,8] they have been shown to extend to anti-platelet aggregation,[9] neurotransmission,[10] immune reactions,[11] and apoptosis.[12]

While RH after temporary ischemia/reperfusion (I/R) is a purposive circulation regulatory mechanism, reperfusion after prolonged ischemia may cause tissue injury, referred to as I/R injury, and has been reported in various tissues including the brain and heart. Previous studies performed pathophysiological analyses on this type of injury.[5] Oxygen free radicals, particularly the superoxide anion (O$_2$$^{\bullet-}$), are now considered to play a crucial role in I/R injury.[13–18] However, the mechanisms underlying I/R injury in the oral cavity region have not yet been elucidated in detail, and the effects of this injury on the regulation of local circulation in the gingiva also remain unclear. Therefore, the aims of the present study were to demonstrate that NO, which is considered to be the key mediator of the local circulation, plays a role in regulating local circulation in the gingiva and to define the effects of I/R on circulatory regulation. The following experimental plans were designed in order to achieve these objectives. 1) We determined whether NO played a role in rat gingival RH pharmacologically. 2) The effects of I/R on NO-related gingival circulatory regulation were examined, and, if present, the mechanisms responsible were elucidated. 3) The prevention and possible treatment of I/R-induced periodontal diseases were investigated.

Materials and Methods

Drugs. N^{ω}-nitro-L-arginine-methyl-ester (L-NAME), 7-nitroindazole (7-NI), superoxide dismutase from bovine erythrocytes (SOD), and hypoxanthine (HX) were purchased from Sigma Chemical Co. (St. Louis, MO), 2,4-diamino-6-hydroxypyrimidine (DAHP) from Aldrich Chemical Co. (Milwaukee, WI), (6R)-5,6,7,8-tetrahydro-L-biopterin (BH$_4$) from Alexis Co. (Laufen, Switzerland), sepiapterin from Cayman Chemical Co. (Ann Arbor, MI), xanthine oxidase (XOD) from Roche Diagnostics Co. (Indianapolis, IN), and 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a] pyrazine-3-one (MCLA, Cypridina Luciferin Analogues) from Tokyo Chemical Industry Co. (Tokyo, Japan). 7-NI was dissolved in dimethyl sulfoxide (DMSO) and diluted with 0.9% physiological saline. L-NAME, DAHP, SOD, BH$_4$, and sepiapterin were dissolved in 0.9% physiological saline when used in *in vivo* experiments. All drugs, except for HX, were prepared on the day of the experiments.

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In *in vivo* experiments, each drug was administered intravenously once.

In *in vitro* chemiluminescence experiments, HX was diluted with pH 7.0 phosphate buffer solution (PBS), and XOD, BH₄, sepiapterin, and MCLA were dissolved in distilled water.

Animals. Male Wistar rats (9–10 weeks old, weighing 250–300 g) were purchased from Japan SLC (Hamamatsu, Japan). The procedures used in this study were in accordance with the guidelines of the US National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication NO. 85-23, revised 1985) and our protocols were approved by the Animal Care Committee of Kanagawa Dental University (Yokosuka, Japan).

Measurement of systemic blood pressure. Under anesthesia with sodium pentobarbital (50 mg/kg, i.p.), each rat was laid on a wooden board (20 × 24 cm) in the supine position. All limbs were fixed at an angle of 45° to the body midline with adhesive tape, and the upper and lower jaws were anchored in an open position with a thin rope via the incisors. Catheters for the administration of drugs were inserted into the femoral vein, while those to measure blood pressure were inserted into the femoral artery. Additional doses of sodium pentobarbital were administered into the femoral vein as needed in order to maintain anesthesia. Blood pressure catheters were filled with 0.9% physiological saline containing heparin (500 U/ml) for the prevention of blood clotting. A pressure transducer was inserted inside the blood pressure catheter. Output signals from the pressure transducer were recorded on a computer hard disc through an A/D converter (PowerLab/8sp, AD Instruments Pty. Ltd., Bella Vista, NSW, Australia) and displayed simultaneously on the monitor. Recorded systemic blood pressure was analyzed using data analysis software (Chart ver. 4.2, AD Instruments, Inc., Colorado Springs, CO.) (Fig. 1).

Measurement of gingival blood flow. Blood flow in the palatal gingiva was measured at randomly selected areas (diameter, 1.0 mm; depth, 1.0 mm) at the palatal gingiva using a laser Doppler flowmeter (ALF21D, ADVANCE Co., Ltd., Tokyo, Japan) with a laser Doppler probe (diameter, 2.0 mm) mounted to an oil hydraulic micromanipulator (Narishige, Tokyo, Japan).

Output signals from the flowmeter were recorded on a computer hard disc through an A/D converter (PowerLab/8sp) and displayed simultaneously on the monitor. Recorded gingival blood flow was analyzed using data analysis software (Chart ver. 4.2) (Fig. 1).

RH protocol. 1) Experiments to evaluate the ischemic time. While the femoral arterial blood pressure was being monitored, RH was generated by applying compression-release directly on the point of the blood flow measurement using a laser Doppler probe. Care was exercised so that tissues were not crushed and constant compression was applied to maintain tissue blood flow at 6 ml/min/100 g. The gingival compression times were 30 s, 60 s, 5 min, 20 min, and 60 min, and were selected randomly. Intervals between compressions were sufficiently long to allow blood flow to return to baseline and become stable. RH was evaluated comparatively by calculating circulating blood volume (mass) in combination with increases in blood flow using data analysis software (Chart ver. 4.2) (Fig. 1).

2) Experiments to suppress RH using various antagonists. The induction of gingival RH following a 60-s compression/reperfusion (control) was compared with that induced 30 min after the treatments with each antagonist (drug treatment groups). The doses of the antagonists used were sufficiently high to significantly suppress the respective agonists, that is, L-NAME (20 mg/kg, i.v.), 7-NI (20 mg/kg, i.v.), and DAHP (20 mg/kg, i.v.).

3) Experiments on I/R. The induction of gingival RH following a 60-s I/R (control) was compared with that induced 20 min after a 60-min compression/reperfusion (I/R group). A single dose each of SOD (36,000 U/kg), BH₄ (10 mg/kg), and sepiapterin (10 mg/kg) was administered intravenously during ischemia 20 min before reperfusion began in I/R, and RH was induced 20 min after reperfusion began in I/R. Each set of treated rats was designated as the SOD group, BH₄ group, and sepiapterin group, respectively, and compared with the I/R group.

Evaluation of O₂·⁻ scavenging activity. In *in vitro* experiments, the O₂·⁻ scavenging activities of drugs (SOD, BH₄, and sepiapterin) were evaluated using the O₂·⁻/MCLA chemiluminescent system. Chemiluminescence intensity was measured using a weak luminescence detection system (Hamamatsu Photonics,
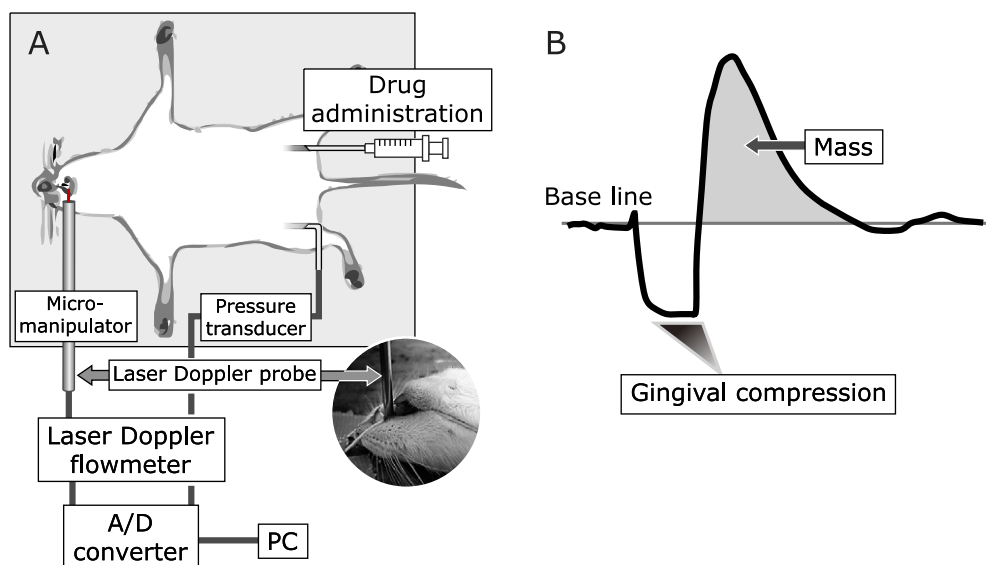
Fig. 1. Schematic diagram of the method used to measure gingival blood flow (GBF) and femoral arterial blood pressure (FBP) (A). The animals were laid on a wooden board in the supine position and limbs were fixed at an angle of 45° to the body midline with adhesive tape. Their upper and lower jaws were anchored in an open position with a thin rope via the incisors. GBF was measured at the palatal gingiva by using a laser Doppler flowmeter with a laser Doppler probe (diameter, 2.0 mm) mounted to an oil hydraulic micromanipulator. Catheters for drug administration were inserted into the femoral vein, while those for measuring blood pressure were inserted into the femoral artery. Typical trace of GBF during RH, illustrating the parameter of mass, integration of increases in GBF during RH (B).

Hamamatsu, Japan). HX (2×10^{-5} M)-XOD (0.1 U/ml) was used for the *in vitro* $O_2^{\cdot-}$ production system, and cypridina luciferin analogues (MCLA), which react specifically with $O_2^{\cdot-}$,[19] were used as chemiluminescent reagents. Distilled water, test drugs (SOD, BH_4, and sepiapterin), HX, and MCLA were added in this order to a chemiluminescent chamber (total volume: 500 μl, 24 wells) and mixed. The chamber was then placed in a shade box. A luminescent reaction was induced by injecting XOD from outside of the shade box and a photon count was performed. The final doses of the test drugs were SOD (0.003, 0.03, 0.3, 3.0, and 30 U/ml), BH_4 (0.1, 1.0, 10.0, 100.0, and 1,000.0 μM), and sepiapterin (0.1, 1.0, 10.0, 100.0, and 1,000.0 μM). Photon counts in 30 s, 30 times, were added to analyze luminescent intensity using image analysis software (AQUACOSMOS, Hamamatsu Photonics, Hamamatsu, Japan), and the results obtained were compared with luminescent intensity in the absence of the test drugs.

In separate experiments, each test drug (SOD, BH_4, and sepiapterin) had 30 and 90 min of contact with the $O_2^{\cdot-}$ production system using HX (10^{-4} M)-XOD (0.5 U/ml), and the $O_2^{\cdot-}$ scavenging activities of the respective test drugs were measured. The final doses of SOD, BH_4, and sepiapterin were prepared to be the same as those in the measurement of $O_2^{\cdot-}$ scavenging activity.

Statistical analysis. All experimental data were expressed as the mean ± SD (n = number of animals). Data comparisons were evaluated using the Student's t test and variance analysis (ANOVA). P values of less than 0.05 ($p < 0.05$) were considered to indicate significant differences.

Results

Ischemic time dependency of RH. Rat gingival blood flow (GBF) decreased immediately following the compression of gingival tissues and blood flow markedly increased following its release (Fig. 2) without affecting systemic blood pressure. Circulatory blood volume (mass) at the time of gingival RH time-dependently increased with a compression time between 30 s and 20 min (Fig. 3). However, the mass of RH following 60 min of compression appeared to be smaller than that with 20 min of ischemia (Fig. 3).

Effects of antagonists on RH. RH induced following a 60-s ischemia was significantly suppressed by the respective pretreatments with L-NAME (20 mg/kg, i.v.), 7-NI (20 mg/kg, i.v.), and DAHP (20 mg/kg, i.v.) (Fig. 4). However, systemic blood pressure significantly increased by 24.7 ± 19.1 and 21.5 ± 21.2% for
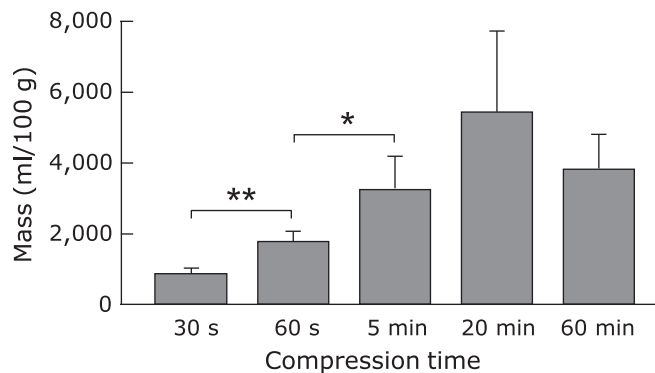

Fig. 3. Relationship between gingival compression times and magnitudes of RH induced by compression of the rat gingiva for 30 s to 60 min. The magnitude of RH was represented as mass (ml/100 g, integration of increases in GBF during RH). Results are expressed as the mean ± SD. *$p<0.05$ and **$p<0.01$ vs the adjoining value. The numbers of experimental animals used were as follows: 30 s, $n = 24$; 60 s, $n = 37$; 5 min, $n = 9$; 20 min, $n = 4$; 60 min, $n = 14$.
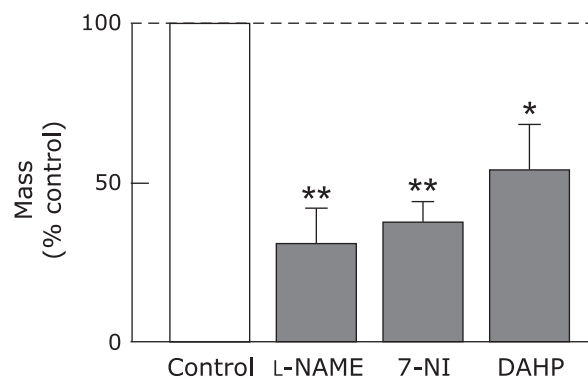
Fig. 4. Effects of pretreatments with L-NAME, 7-NI, and DAHP on RH induced by compression for 60 s. The vertical axis represents the relative mass value (mass: ml/100 g, integration of increases in GBF during RH) when the mass value of the untreated control group was estimated as 100% (% control). Results are expressed as the mean ± SD. *$p<0.05$ and **$p<0.01$ vs control values. The number of experimental animals used was 5.
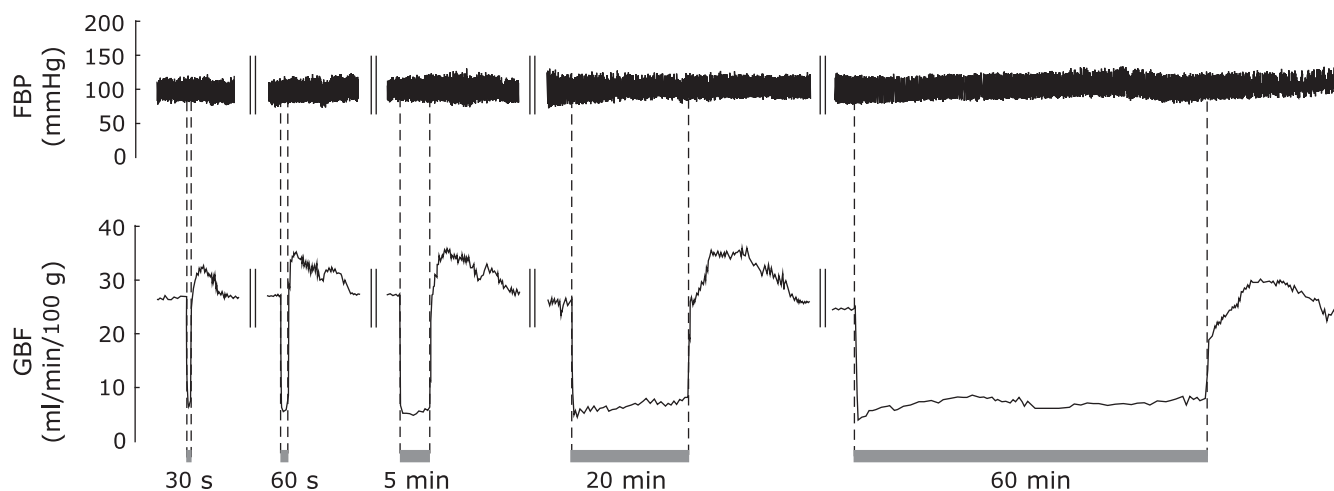
Fig. 2. Recordings of femoral arterial blood pressure (FBP) and gingival blood flow (GBF) when compression was applied to the rat gingiva between 30 s and 60 min. Horizontal lines represent the compression time.
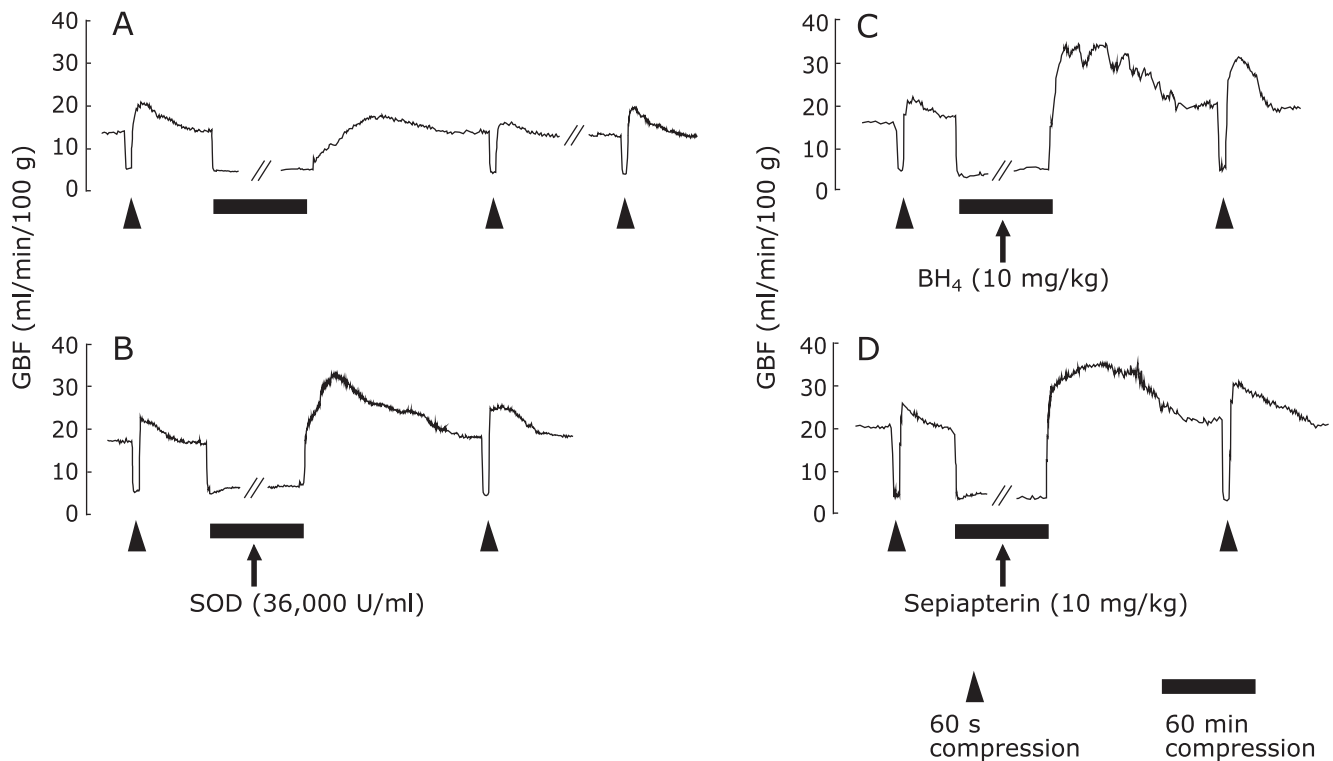
Fig. 5. Typical recordings of gingival RH induced by compression for 60 s (closed triangle) before and after a 60-min ischemia/reperfusion (I/R) (A), and effects of SOD (B), BH₄ (C), and sepiapterin (D) administered intravenously (arrow) during ischemia 20 min before reperfusion in I/R.

30 min after the pretreatments with L-NAME and 7-NI, respectively, while the change observed in systemic blood pressure with DAHP (20 mg/kg, i.v.) was $1.3 \pm 3.9\%$ and not significant.

**Effects of I/R induced by compression for 60 min on RH.**
RH induced by compression for 60 s was markedly inhibited after I/R induced by compression for 60 min (Fig. 5). However, this inhibition was temporary and disappeared 60 min after reperfusion (Fig. 5A). Furthermore, this inhibition was significantly decreased (Fig. 6) by the pretreatments with SOD (36,000 U/kg), BH₄ (10 mg/kg), and sepiapterin (10 mg/kg). Blood flow in I/R and subsequent RH increased (Fig. 6). Systemic blood pressure decreased slightly by 7.0 ± 9.7, 1.6 ± 6.6, and $5.1 \pm 3.7\%$ following the pretreatments with SOD, BH₄, and sepiapterin, respectively.

Evaluation of $O_2^{\bullet-}$ scavenging activity. SOD, BH₄, and sepiapterin dose-dependently decreased the luminescent intensity of $O_2^{\bullet-}$-induced MCLA. SOD and BH₄, in particular, exhibited strong $O_2^{\bullet-}$ scavenging activities (Fig. 7). The IC_{50}, or 50% inhibition concentrations, of SOD, BH₄, and sepiapterin were 0.4 U/ml, 10 μM, and 1 mM, respectively. When BH₄ had contact with the $O_2^{\bullet-}$ production system for 30 and 90 min in advance, it significantly decreased $O_2^{\bullet-}$ scavenging activity (Fig. 8). However, neither SOD nor sepiapterin had any effects on $O_2^{\bullet-}$ scavenging activity (Fig. 8).

Discussion

Gingival RH time-dependently increased with compression when the compression time was between 30 s and 20 min (Fig. 2 and 3). RH was significantly suppressed by the non-selective NOS inhibitor L-NAME, selective nNOS inhibitor 7-NI, and BH₄ (NOS co-factor) production inhibitor DAHP (Fig. 4). These results suggested that neurogenic NO played an important role in vasodilatation in rat gingival RH.

We previously demonstrated using NO electrodes that NO
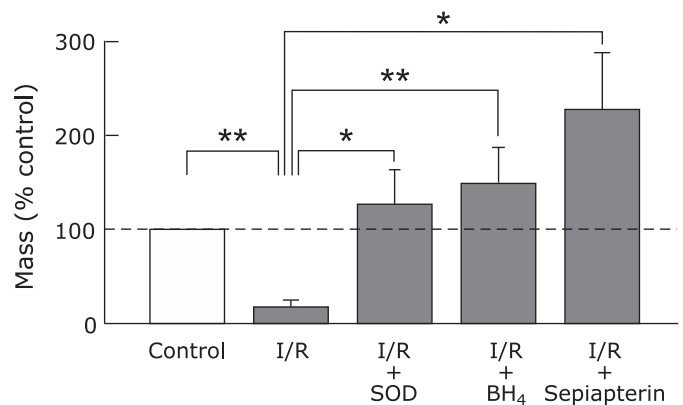
Fig. 6. Irreparable effects of SOD, BH₄, and sepiapterin on I/R-induced inhibition of RH. The vertical axis represents the relative mass value (mass: ml/100 g, integration of increases in GBF during RH) when the mass value of the untreated control group was estimated as 100% (% control). Results are expressed as the mean ± SD. *$p<0.05$ and **$p<0.01$ vs the value of I/R. The numbers of experimental animals used were as follows: control group (open column), $n = 6$; I/R group, $n = 6$; SOD group, $n = 5$; BH₄ group, $n = 6$; sepiapterin group, $n = 4$.

directly played a role in dog gingival RH; the NO current was slowly elevated in dog gingival tissues during ischemia.[2] RH subsequently occurred in parallel with a marked elevation in the NO current. RH was not affected by cholinergic blocking agents, adrenergic blocking agents, or antihistaminic agents, and was markedly suppressed by L-NAME. These results indicated that NO played a major role in RH as a non-cholinergic, non-adrenergic, and non-histaminergic regulatory agent.[2,3] Previous findings obtained in dog gingiva were consistent with our results
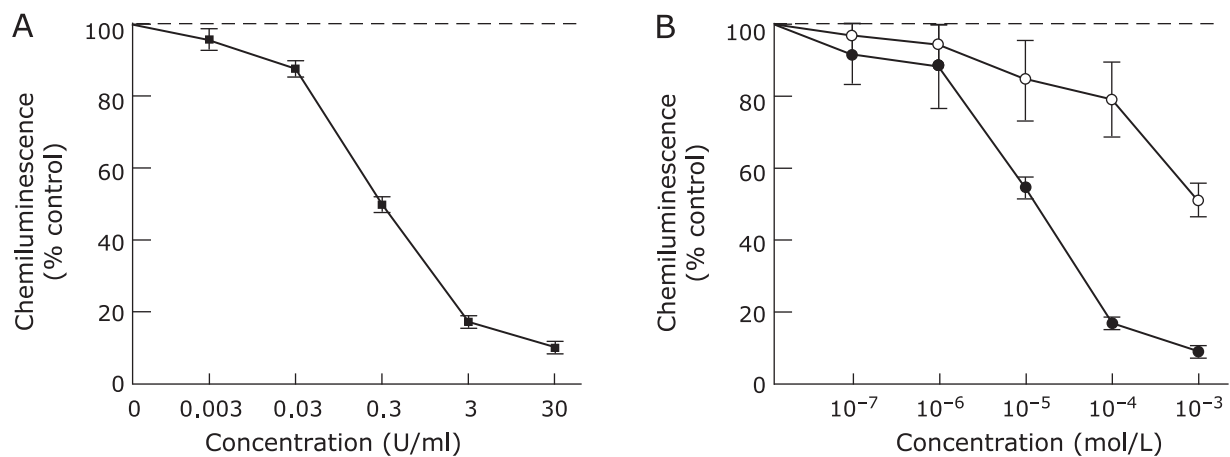
Fig. 7. Relationship between $O_2^{\bullet-}$ scavenging activity and concentrations of SOD (A, closed square), BH_4 (B, closed circle), and sepiapterin (B, open circle). The vertical axis represents the relative chemiluminescence intensity when the mass value of the untreated control group was estimated as 100%. Each scavenging activity was represented as the decrease in MCLA chemiluminescent intensity (CL, % control) induced by the *in vitro* $O_2^{\bullet-}$ generating system (HX plus XO). Results are expressed as the mean $\pm$ SD ($n = 5$).
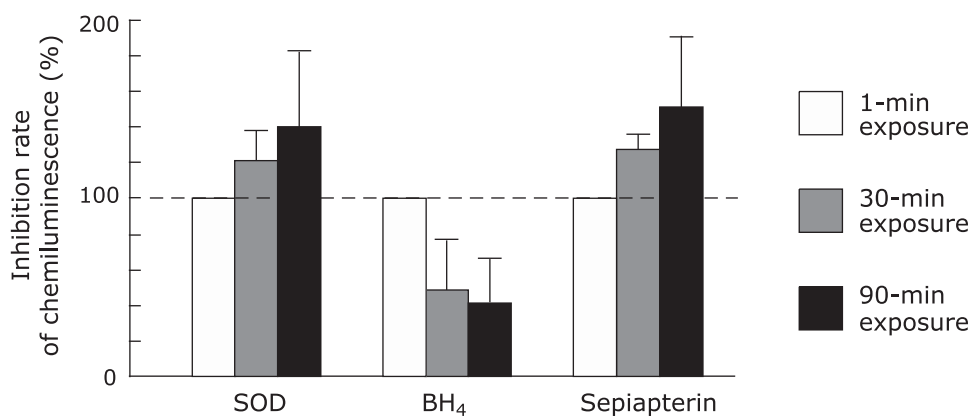
Fig. 8. Effects of exposure to the $O_2^{\bullet-}$ generating system on scavenging activities of SOD, BH_4, and sepiapterin. SOD, BH_4, and sepiapterin (IC_{50} of CL) were exposed to the *in vitro* $O_2^{\bullet-}$ generating system (HX plus XO) for 1, 30, or 90 min, and $O_2^{\bullet-}$ scavenging activities were subsequently evaluated. The inhibition rates of the IC_{50} of SOD, BH_4, and sepiapterin (1-min exposure), indicated as 100% (open column), were compared with those of SOD, BH_4, and sepiapterin (hatched column, 30-min exposure; black column, 90-min exposure). Results are expressed as the mean $\pm$ SD ($n = 3$).

on the suppression of rat gingival RH. Toda *et al.*[20] showed that the nitrergic nerve acted as a vasodilator. Lohinai *et al.*[21] detected the presence of the nitrergic nerve in rat oral cavity tissues such as the gingiva and dental pulp using histochemical procedures. Therefore, NO secreted by the nitrergic nerve as a paracrine agent is considered to play a role in rat gingival RH. The method used to apply vascular avascularization and release including RH in the present study is also applied as flow-mediated dilation (FMD) and is considered suitable for non-invasive assessments of vascular endothelial function and stiffness via NO and other vasodilators. Furthermore, this method has already been applied to humans in clinical settings for the early detection and treatment of lifestyle-related diseases such as hypertension, arterial sclerosis, and diabetes. Non-invasive techniques have recently been developed to assess endothelial function in humans,[22] are typically applied to the arm or forearm, and evaluate endothelial function in the brachial artery or resistance vessels of the upper arm.[23–25] These methods determine RH (a physiological response) by occlusion/release with the cuff of a sphygmomanometer. Evidence in the field of dentistry to support gingival RH being measurable by laser Doppler flowmeter is accumulating,[2,3,26] and this response is proportional to the level of lifestyle-related diseases linked to vascular endothelial function, similar to the method using the brachial artery.[26]

NO is generally produced in endothelial cells. NO released from endothelial cells through a stimulus such as shear stress[27] or endogenous endothelial-relaxing factor[28] easily permeates through cellular membrane and diffuses to the smooth muscle layer directly below endothelial cells. NO activates soluble guanylyl cyclase in the cytoplasm, produces cGMP, a second messenger in the intracellular signal transmission system, from GTP and finally relaxes smooth muscles through the phosphorylation of a G-kinase-related protein.[7,8] Although we did not determine whether NO originated from endothelial cells or nerves in the present study, ischemia-induced time-dependent increases in RH up to 20 min were attributed to increases in NOS activity in proportion to tissue ischemia and the corresponding amount of NO produced. The mechanism underlying the release of NO with an ischemic stimulation has not yet been elucidated in detail. Henrich *et al.*[29] previously reported that the release of NO from rat and mouse sensory nerve cells was triggered by the influx of Ca^{2+} into cells. Ischemia has been suggested to decrease intra-

cellular ATP levels and suppress the sodium pump, which is necessary for the maintenance of nervous function, thereby elevating intracellular Na^+ levels. Abnormally elevated intracellular Na^+ levels reverse the Na^+/Ca^{2+} exchanger, generating Na^+ outflow and simultaneous Ca^{2+} influx. Increases in intracellular Ca^{2+} levels, together with calmodulin, have been shown to activate NOS, thereby facilitating the production of NO.[30,31]

Rat gingival RH was previously reported to be markedly inhibited by a pretreatment with DAHP, a selective inhibitor of GTP cyclohydrolase 1 (GTPCH 1),[32] a rate-limiting enzyme associated with the production of BH_4 from GTP, indicating that NOS activity is essential for gingival RH and also that BH_4 plays an important role in hyperemia.

Another aim of the present study was to determine the effects of I/R on the regulatory system of gingival local circulation. $O_2^{•-}$ produced in I/R is an oxygen free radical that is now attracting attention as a key factor and initiator of I/R injury.[33] In the present study, gingival-RH induced by compression for 60 s was markedly inhibited after I/R induced by compression for 60 min (Fig. 5). This inhibition was significantly recovered by the treatments with SOD, BH_4, and sepiapterin, indicating a strong relationship with $O_2^{•-}$ (Fig. 5 and 6). In the present study, RH induced by compression from 30 s to 20 min increased in a time-dependent manner, thereby reflecting a physiological response, while I/R induced by compression for 60 min and release for 20 min mimicked a non-physiological pathological stimulation. RH induced by compression for 60 min was lower than that for 20 min, and time dependency was not observed (Fig. 3). Therefore, the RH induced by compression for 60 s after I/R caused by ischemia for 60 min is a response after 60-min exposure to non-physiological environments, such as oxidative stress. Furthermore, drug treatments were administered during the I/R period in order to determine their effects on the non-physiological environment including oxidative stress. The reduction observed in RH after I/R and chemiluminescence of $O_2^{•-}$ by MCLA were inhibited by BH_4 to a similar extent to that by SOD (Fig. 7). The results of the present study suggested that BH_4 inhibited the suppression of RH induced by compression for 60 s after I/R induced by compression for 60 min and release for 20 min in the rat gingiva. $O_2^{•-}$ sources have been suggested to originate in the HX/XOD system,[34] NADPH oxidase system,[35] and NOS,[36] but were not identified in the present study. The inhibition of increases in blood flow in I/R were previously reported to be markedly decreased by the pretreatments with BH_4 and its precursor sepiapterin.[37] BH_4 is a co-factor of NOS that plays an important role in the electronic transmission system in the NO production process from L-arginine.[38] As shown in Fig. 5 and 6, decreases in gingival RH levels that were recovered by the administration of BH_4 during the 60-min period of ischemia indicated that the target of $O_2^{•-}$ may not be an enzyme system such as GTPCH 1, but easily producible BH_4. In other words, the temporary depletion of BH_4 by $O_2^{•-}$ decreased NOS activity, ultimately resulting in the inhibition of RH. Therefore, when sufficient reperfusion decreases intracellular $O_2^{•-}$ and BH_4 production exceeds its consumption by $O_2^{•-}$, the production of NO returns, and RH gradually recovers. This is consistent with the inhibition by I/R being temporary, inhibition recovering in 60 min, and BH_4, which exhibited strong $O_2^{•-}$ scavenging activity in MCLA chemiluminescence experiments, losing its activity through self-destruction (Fig. 7 and 8).

BH_4 is used clinically in substitution therapy for a BH_4 deficiency, such as that in hyperphenylalaninemia.[39] The present study demonstrated its possible application in dental treatments, for example, incompatible dentures cause intermittent I/R at the gingiva, which may lead to the formation of ulcers. Decubitus ulcers are well-known I/R injuries at the skin.[40] BH_4 and sepiapterin may be effective agents for preventing and improving I/R-induced periodontal diseases.

In summary, the results of the present study suggested that NO secreted by the nitrergic nerve as a paracrine agent played a role in local circulatory regulation in rat gingival RH. The NO-related regulation of the local circulation was temporarily inhibited by gingival I/R treatments. The decrease observed in NO production, which was caused by the suppression of NOS activity subsequent to the depletion of the NOS co-factor BH_4 by $O_2^{•-}$, may have played a partial role in this inhibition.

Acknowledgments

Conflict of Interest

No potential conflicts of interests were disclosed.

References

1 Bache RJ, Cobb FR, Greenfield JC Jr. Limitation of the coronary vascular response to ischemia in the awake dog. *Circ Res* 1974; **35**: 527–535.

2 Shimada S, Todoki K, Omori Y, *et al*. Contribution of nitrergic nerve in canine gingival reactive hyperemia. *J Clin Biochem Nutr* 2015; **56**: 98–104.

3 Omori Y, Takahashi S, Todoki K. Role of nitric oxide in post-ischemic gingival hyperemia in anesthetized dogs. *Redox Report* 2002; **7**: 300–303.

4 Meredith IT, Currie KE, Anderson TJ, Roddy MA, Ganz P, Creager MA. Postischemic vasodilation in human forearm is dependent on endothelium-derived nitric oxide. *Am J Physiol* 1996; **270 (4 Pt 2)**: H1435–H1440.

5 Otomo J, Nozaki N, Tomoike H. Roles of nitric oxide and adenosine in the regulation of coronary conductance in the basal state and during reactive hyperemia. *Jpn Circ J* 1997; **61**: 441–449.

6 Shibata M, Araki N, Hamada J, Sasaki T, Shimazu K, Fukuuchi Y. Brain nitrite production during global ischemia and reperfusion: an *in vivo* micro-dialysis study. *Brain Res* 1996; **734**: 86–90.

7 Palmer RM, Rees DD, Ashton DS, Moncada S. L-Arginine is the physio-logical precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem Biophys Res Commun* 1988; **153**: 1251–1256.

8 Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 1987; **84**: 9265–9269.

9 Shen YC, Hong CY. Effect of trilinolein on cyclic nucleotide formation in human platelets: relationship with its antiplatelet effect and nitric oxide synthesis. *Br J Pharmacol* 1995; **116**: 1644–1648.

10 Paakkari I, Lindsberg P. Nitric oxide in the central nervous system. *Ann Med* 1995; **27**: 369–377.

11 Liu HW, Anand A, Bloch K, Christiani D, Kradin R. Expression of inducible nitric oxide synthase by macrophages in rat lung. *Am J Respir Crit Care Med* 1997; **156**: 223–228.

12 Brüne B, von Knethen A, Sandau KB. Nitric oxide and its role in apoptosis. *Eur J Pharmacol* 1998; **351**: 261–272.

13 Kinouchi H, Epstein CJ, Mizui T, Carlson E, Chen SF, Chan PH. Attenuation of focal cerebral ischemic injury in transgenic mice overexpressing CuZn superoxide dismutase. *Proc Natl Acad Sci U S A* 1991; **88**: 11158–11162.

14 Hoshida S, Kuzuya T, Yamashita N, *et al*. Brief myocardial ischemia affects free radical generating and scavenging systems in dogs. *Heart Vessels* 1993; **8**: 115–120.

15 Yoshikawa T, Naito Y, Ueda S, *et al*. Role of oxygen-derived free radicals in the pathogenesis of gastric mucosal lesions in rats. *J Clin Gastroenterol* 1990; **12 Suppl 1**: S65–S71.

16 Grisham MB, Hernandez LA, Granger DN. Xanthine oxidase and neutrophil infiltration in intestinal ischemia. *Am J Physiol* 1986; **251 (4 Pt 1)**: G567–G574.

17 Okuda M, Ikai I, Chance B, Kumar C. Oxygen radical production during ischemia-reperfusion in the isolated perfused rat liver as monitored by luminal enhanced chemiluminescence. *Biochem Biophys Res Commun* 1991;

174: 217–221.

18 Galat JA, Robinson AV, Rhodes RS. Oxygen free radical mediated renal dysfunction. *J Surg Res* 1989; **46**: 520–525.

19 Nakano M. Detection of active oxygen species in biological systems. *Cell Mol Neurobiol* 1998; **18**: 565–579.

20 Toda N, Okamura T. The pharmacology of nitric oxide in the peripheral nervous system of blood vessels. *Pharmacol Rev* 2001; **55**: 271–324.

21 Lohinai Z, Székely AD, Benedek P, Csillag A. Nitric oxide synthase containing nerves in the cat and dog dental pulp and gingival. *Neurosci Lett* 1997; **227**: 91–94.

22 Lehmann ED, Riley WA, Clarkson P, Gosling RG. Non-invasive assessment of cardiovascular disease in diabetes mellitus. *Lancet* 1997; **350 Suppl 1**: SI14–SI19.

23 Ochiai R, Sugiura Y, Otsuka K, Katsuragi Y, Hashiguchi T. Coffee bean polyphenols ameliorate postprandial endothelial dysfunction in healthy male adults. *Int J Food Sci Nutr* 2015; **66**: 350–354.

24 Ito H, Nakashima M, Meguro K, *et al*. Flow mediated dilatation is reduced with the progressive stages of glomerular filtration rate and albuminuria in type 2 diabetic patients without coronary heart disease. *J Diabetes Res* 2015; **2015**: 728127.

25 Nezu T, Hosomi N, Aoki S, *et al*. Endothelial dysfunction is associated with the severity of cerebral small vessel disease. *Hypertens Res* 2015; **38**: 291–297.

26 Sugiyama S, Takahashi S, Tokutomi F, *et al*. Gingival vascular functions are altered in type 2 diabetes mellitus model and/or periodontitis model. *J Clin Biochem Nutr* 2012; **51**: 108–113.

27 Schoen JM, Wang HH, Minuk GY, Lautt WW. Shear stress-induced nitric oxide release triggers the liver regeneration cascade. *Nitric Oxide* 2001; **5**: 453–464.

28 Furchgott RF. Introduction to EDRF research. *J Cardiovasc Pharmacol* 1993; **22 Suppl 7**: S1–S2.

29 Henrich M, Hoffmann K, König P, *et al*. Sensory neurons respond to hypoxia with NO production associated with mitochondria. *Mol Cell Neurosci* 2002;

20: 307–322.

30 Presta A, Liu J, Sessa WC, Stuehr DJ. Substrate binding and calmodulin binding to endothelial nitric oxide synthase coregulate its enzymatic activity. *Nitric Oxide* 1997; **1**: 74–87.

31 Zubrow AB, Delivoria-Papadopoulos M, Ashraf QM, Fritz KI, Mishra OP. Nitric oxide-mediated Ca^{2+}/calmodulin-dependent protein kinase IV activity during hypoxia in neuronal nuclei from newborn piglets. *Neurosci Lett* 2002; **335**: 5–8.

32 Nichol CA, Smith GK, Duch DS. Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. *Annu Rev Biochem* 1985; **54**: 729–764.

33 Granger DN. Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. *Am J Physiol* 1988; **255 (6 Pt 2)**: H1269–H1275.

34 Ortiz PA, Garvin JL. Superoxide stimulates NaCl absorption by the thick ascending limb. *Am J Physiol Renal Physiol* 2002; **283**: F957–F962.

35 Sankarapandi S, Zweier JL, Mukherjee G, Quinn MT, Huso DL. Measurement and characterization of superoxide generation in microglial cells: evidence for an NADPH oxidase-dependent pathway. *Arch Biochem Biophys* 1998; **353**: 312–321.

36 Vásquez-Vivar J, Kalyanaraman B. Generation of superoxide from nitric oxide synthase. *FEBS Lett* 2000; **481**: 305–306.

37 Shimizu S, Ishii M, Kawakami Y, Momose K, Yamamoto T. Protective effects of tetrahydrobiopterin against nitric oxide-induced endothelial cell death. *Life Sci* 1998; **63**: 1585–1592.

38 Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J* 2001; **357**: 593–615.

39 Laufs S, Blau N, Thony B. Retrovirus-mediated double transduction of the GTPCH and PTPS genes allows 6-pyruvoyltetrahydropterin synthase-deficient human fibroblasts to synthesize and release tetrahydrobiopterin. *J Neurochem* 1998; **71**: 33–40.

40 Sundin BM, Hussein MA, Glasofer S, *et al*. The role of allopurinol and deferoxamine in preventing pressure ulcers in pigs. *Plast Reconstr Surg* 2000; **105**: 1408–1421.