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Breathing Nitric Oxide plus Hydrogen Gas Reduces Ischemia-Reperfusion Injury and Nitrotyrosine Production in Murine Heart

Toshihiro Shinbo,[1] Kenichi Kokubo,[1,2] Yuri Sato,[2] , Shintaro Hagiri,[3] Ryuji Hataishi,[3] Minoru Hirose,[1,2] Hirosuke Kobayashi,[1,2]

From the [1]Department of Medical Engineering and Technology, Kitasato University School of Allied Health Sciences; [2]Kitasato University Graduate School of Medical Sciences; and [3]Department of Medicine, Kitasato University School of Medicine, Kanagawa, Japan

Running Head: **Inhalation of NO and H_2 Reduces Heart I-R Injury**

Contact Information:
Hirosuke Kobayashi, MD, PhD
Kitasato University Graduate School of Medical Sciences, Kitasato 1-15-1, Minami-ku, Sagamihara-shi, Kanagawa 252-0373, Japan
Tel/Fax +81(0)42-778-9973
Email hiro@kitasato-u.ac.jp

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Abstract

Inhaled nitric oxide (NO) has been reported to decrease the infarct size in cardiac ischemia reperfusion (I-R) injury. However, reactive nitrogen species (RNS) produced by NO causes myocardial dysfunction and injury. Since H_2 is reported to eliminate peroxynitrite, it was expected to reduce the adverse effects of NO. In mice, left anterior descending coronary artery ligation for 60 min followed by reperfusion was performed with inhaled NO (80 ppm), H_2 (2%), or NO + H_2, starting 5 min before reperfusion for 35 min. After 24 hrs, left ventricular function, the infarct size and area at risk (AAR) were assessed. Oxidative stress associated with reactive oxygen species (ROS) was evaluated by staining for 8-hydroxy-2'-deoxyguanosine and 4-hydroxy-2-nonenal, that associated with RNS by staining for nitrotyrosine, and neutrophil infiltration by staining for granulocyte receptor-1. The infarct size/AAR decreased with breathing NO or H_2 alone. NO inhalation plus H_2 reduced the infarct size/AAR, with significant interaction between the two, reducing ROS and neutrophil infiltration, and improved the cardiac function to normal levels. While nitrotyrosine staining was prominent after NO inhalation alone, it was eliminated after breathing a mixture of H_2 with NO. Preconditioning with NO significantly reduced the infarct size/AAR, but not preconditioning with H_2. In conclusion, breathing NO + H_2 during I-R reduced the infarct size and maintained cardiac function, and reduced the generation of myocardial nitrotyrosine associated with NO inhalation. Administration of NO + H_2 gases for

47 inhalation may be useful for planned coronary interventions or for the treatment of I-R injury.

48

49 (250 word)

50 **Keywords:** nitric oxide, hydrogen gas, antioxidants, ischemia, reperfusion injury

51

Introduction

It is well-known that nitric oxide (NO) can produce both desirable and undesirable effects (25, 26): NO has been reported to have both anti-inflammatory and cytocidal effects. The anti-inflammatory effects are presumably mediated by inhibition of platelet and neutrophil activation *via* enhanced guanylate cyclase (17) and/or poly (ADP-ribose) polymerase activity (3), and the cytocidal effects are presumably mediated by reactive nitrogen species (RNS), such as peroxynitrite generation (2), or from alternative pathways including nitrite/H_2O_2/hemeperoxidase and transition metal-dependent mechanisms (20).

Tyrosine nitration caused by RNS or the alternative pathways increases the activity of cytochrome C, fibrinogen and protein kinase Cε, and decreases the activity of mitochondrial manganese superoxide dismutase, prostacyclin synthase, actin (20) and hemoxygenase (12). It might have desirable effects in some specific situations (5), but the reactions of RNS or the alternative pathways are strong and uncontrollable, and may cause cellular malfunction, necrosis or apoptosis (2, 5), resulting in organ failure.

Inhaled NO has recently been reported to produce a wide range of extra-pulmonary effects, such as inhibition of platelet and neutrophil activation, vasodilatation in ischemic tissues, and so on (6, 15). Geury *et al.* then demonstrated that pretreatment with inhaled 10 ppm NO improved cardiac function after I-R (8), and Hataishi *et al.* reported that inhaled NO decreased infarct size in mice subjected to cardiac ischemia followed by reperfusion (9).

They noted that NO inhalation of 40 or 80 ppm, but not 20 ppm decreased infarct size and improved the cardiac function. In the pig, inhaled 80 ppm NO was reported to decrease infarct size and improve cardiac function after myocardial I-R (13). These reports indicated the possible clinical usefulness of NO inhalation during coronary intervention, and a phase 2 randomized clinical trial is now under way: Effects of nitric oxide for inhalation in myocardial infarction size (NOMI), registered as NCT01398384 on July 18, 2011, refer to http://clinicaltrials.gov/ct2/show/NCT01398384.

In 2008 Ohsawa *et al.* reported that hydrogen gas (H_2) has the potential to act as an antioxidant (19). In their study, it was shown that H_2 selectively reduced the generation of hydroxyl radicals and peroxynitrite, thereby protecting the cells against oxidant injury. Furthermore, they studied an acute rat model in which oxidative stress was induced in the brain by focal I-R, and inhaled H_2 gas markedly suppressed the associated brain injury. Thus, it was suggested that administration of H_2 by inhalation may serve as an effective therapy for I-R, and based on the ability of H_2 gas to rapidly diffuse across membranes, it can even protect ischemic tissues against oxidative damage. Hayashida *et al.* investigated whether inhaled H_2 gas conferred cardioprotection against myocardial I-R injury in rats, and in an *in vivo* study, they observed that inhaled H_2 gas was rapidly transported to 'at risk' ischemic myocardium before coronary blood flow was reestablished in the occluded region (10). In the dogs, inhaled 1.3% H_2 gas was also reported to decrease infarct size after myocardial I-R (30).

These reports indicated the possible clinical usefulness of H_2 inhalation during coronary intervention, and a randomized clinical trial is now in preparation for recruiting: The safety and efficacy of inhalation of H_2 gas during PCI in patients with acute myocardial infarction, registered as UMIN000006825 on December 4, 2011.

Reasoning from these previous reports, we hypothesized that the inhibitory effect of NO on inflammation may be enhanced by eliminating highly reactive by-products of NO inhalation, such as peroxynitrite, by adding H_2 gas to inhaled NO gas. NO exerts potent inhibitory effects on platelets and neutrophils *via* activation of guanylate cyclase, and is expected to dilate small blood vessels, particularly in ischemic tissues.

The aim of the present study was to determine whether inhalation of NO combined with H_2 gas might be more effective at reducing infarct size and improving heart function in a murine model of myocardial I-R in comparison to inhalation of NO or H_2 alone.

Our findings indicate that breathing NO plus H_2 can reduce cardiac injury and augment recovery of the left ventricular function, by elimination of the nitrotyrosine produced by NO inhalation alone, and suggest the potential usefulness of employing a mixture of NO plus H_2 gases for inhalation during planned coronary interventions or for the treatment of I-R injury.

Materials and Methods

Experimental procedures (Fig. 1)

109 The animal experimental protocol was approved by the Animal Research Committee of
110 Kitasato University School of Allied Health Sciences as well as of Medicine, and conformed
111 to the ‘‘Guide for the Care and Use of Laboratory Animals’’ provided by the Institute for
112 Laboratory Animal Research (National Academy of Sciences) in 1996; the animal facility met
113 the standards of the American Association for Accreditation of Laboratory Animal Care.
114 Ten-week-old male C57BL/6J mice (body weight: 20-25 g) (CLEA Japan Inc, Tokyo,
115 Japan) were anesthetized by intraperitoneal administration of pentobarbital (60 mg/kg) and
116 ventilated (MiniVent, Hugo Sachs Elektronik, Harvard Apparatus, Holliston, MA, USA) at
117 F_{IO_2} 0.2. Following thoracotomy, myocardial ischemia was induced by ligation of the left
118 coronary artery at the level of the left atrial appendage for 60 min at F_{IO_2} 0.3, followed by
119 reperfusion for 24 h. From 5 min before reperfusion to 30 min after reperfusion, 2% H_2, 80
120 ppm NO, or a mixture of NO (80 ppm, 0.008%) and H_2 (2%) was administered by inhalation.
121 The thorax was then closed in layers, and animals were allowed to recover from anesthesia.
122 Ventilation at F_{IO_2} 0.3 was continued until the mice were awake. After disconnecting from the
123 ventilator and extubation of the trachea, mice were transferred into clean cages with free
124 access to food and water.
125 NO was given at a concentration of 80 ppm, based upon a previous study on the
126 cardioprotective effect of NO after myocardial I-R injury in mice, where NO inhalation of 40
127 or 80 ppm, but not 20 ppm, decreased infarct size and improved the cardiac function (9). H_2

was given at a concentration of 2%, based upon our dose-response experiment, where we confirmed that inhalation of 2% H_2 reduced murine myocardial I-R injury and that the effect did not significantly differ from the effect of breathing 3% H_2 (see Results shown below).

Previous studies have also reported that 2% H_2 inhalation was effective against I-R injury of the brain (19), liver (7) and heart (10) in rats; furthermore, it was confirmed that the H_2 levels in the blood and myocardium changed within a few minutes of starting and discontinuing 2% H_2 inhalation, and H_2 at a concentration of less than 4% in air is neither flammable nor combustible (1).

In an attempt to eliminate the variable effects of H_2 produced by the natural enterobacterial flora, animals were administered antibiotics for 4 days by mixing penicillin (1.25 mg/ml) and streptomycin (2 mg/ml) in the drinking water, and were denied access to food for 18 hr before the surgery, and mice not treated with the antibiotics and with free food access were also studied as a control. To exclude the possible effects of antibiotics themselves on the infarct size after I-R, the antibiotics were injected intramuscularly just before the I-R experiments, and ratios of area at risk (AAR) to the total area of the left ventricule (LV) and the infarct size to AAR (infarct size /AAR) were observed.

Dose response curve of breathing H_2 gas concentration and infarct size

The dose dependency of inhaled H_2 ranging from 1% to 3% H_2 on infarct size (see

147 below: **Measurement of myocardial infarct size**) in mice without treatment of antibiotics
148 and fasting was examined in reference to the area at risk.
149
150 **Measurement of H_2 concentration in the expired air of mice**
151 Before surgery, expired H_2 concentrations were measured with an H_2 sensor (XP-3160,
152 New Cosmos Electric Co Ltd., Osaka, Japan). Expired air of mice was forced to flow at 50
153 ml/min for 10 min into a gas-tight sampling bag made of aluminum. The expired air in the
154 bag was then introduced into the H_2 sensor (Fig. 2A). We confirmed that the H_2 concentration
155 in the expired air of antibiotics-treated mice was below the 1 ppm detection limit, while it
156 was well over the detection limit in all of the antibiotics non-treated mice (Fig. 2B).
157
158 **Echocardiography**
159 Transthoracic echocardiography was performed with a commercially available
160 echocardiography system (ProSound SDD-4000; ALOKA Co, Ltd, Tokyo, Japan), using a
161 4-14 MHz linear-array transducer. Closed-chest echocardiograms were obtained under
162 anesthesia before the surgery and 24 h after reperfusion. The left ventricular ejection fraction
163 (LVEF) was calculated using M-mode echocardiography.
164
165 **Measurement of myocardial infarct size**

166 At 24 h after reperfusion, the left coronary artery was re-ligated, and a tissue-marking
167 dye (Evans blue, Wako Pure Chemical, Tokyo, Japan) was injected through the inferior *vena*
168 *cava* to determine the area at risk (AAR). After the heart was harvested and placed in an
169 agarose-containing well, the heart was sliced into 6 to 7 transverse slices of 1-mm thickness.
170 The slices were placed in 1% 2,3,5-triphenyltetrazolium chloride solution in PBS for 10 min
171 (first stain) and for 5 min (second stain), then washed with phosphate-buffered saline for 5
172 min and fixed with formalin for 10 min. The infarct zone remained pale yellow, whereas the
173 viable areas stained deep red. Digital images of the slices were obtained. The area stained
174 with neither Evans blue (area at risk, AAR) nor 2,3,5-triphenyltetrazolium chloride (infarct
175 size) was measured on each slice image using an image software (Image J, NIH, USA), and
176 summed. Ratios of AAR to the total area of the LV and the infarct size to AAR (infarct size
177 /AAR) were calculated.
178
179 **Immunohistochemical staining**
180 Immunohistochemical staining of the heart tissues for 8-hydroxy-2'-deoxyguanosine
181 (8-OHdG), 4-hydroxy-2-nonenal (4HNE) and nitrotyrosine, and staining for granulocyte
182 receptor-1 (Gr-1) of the neutrophils in the heart at 24 h after reperfusion were carried out (at
183 the Biopathology Institute Co., Ltd, Oita, Japan, for the 8-OHdG, 4HNE and nitrotyrosine
184 stainings, and at Applied Medical Research Laboratory, Osaka, Japan, for the Gr-1 staining)

with randomized ID numbers, to ensure that the institute technicians were blind to the treatment allocation. Each harvested heart was fixed in Bouin's solution (picric acid-saturated solution : formalin : acetic acid = 15 : 5 : 1) for 3 days. Paraffin sections were prepared using a Young-type sliding microtome (Sakura Finetek Japan, Co., Ltd.), with a disposable microtome blade. The sections were 4 μm thick and mounted on silane-coated slides and deparaffinized with xylene, followed by stepwise changes of the ethanol concentration.

The slides for 8-OHdG, 4HNE and nitrotyrosine staining were treated with 1% hydrogen peroxide/methanol for 30 min to block endogenous peroxidase activity, and rinsed in Tris-buffered saline. Next, they were treated with 8% skimmed milk for 30 min. Anti-8-OHdG monoclonal antibody, anti-4HNE monoclonal antibody (each diluted 1:50, Japan Institute for the Control of Aging, Nikken Seil Co., Ltd : JaICA Japan) and anti-nitrotyrosine polyclonal antibody (diluted 1:200; 5 μg/mL diluted in phosphate-buffered saline containing 1% bovine serum albumin, Millipore, USA) in Tris-buffered saline were applied overnight to the sections in a moisture chamber at 4ºC. They were then incubated in streptavidin-biotin-peroxidase solution according to the instructions for the strept ABComplex/HRP kit (Dako Japan Co., Ltd., Japan) for 8-OHdG and 4HNE staining, or according to the instructions for the MAX-PO kit (NICHIREI, Japan) for nitrotyrosine staining. The immunoreaction was visualized by the 3,3- diaminobenzidine (DAB) reaction for 8-OHdG and 4HNE, and 3-amino-9-ethylcarbazole (AEC) for nitrotyrosine. The sections

were counterstained with hematoxylin.

The slides for Gr-1 were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, and rinsed in distilled water and Tris-buffered saline containing Tween 20. Next, they were treated with blocking solution containing 0.25% casein in PBS, stabilizing protein and 0.015 mol/L sodium azide (X0909, Dako Japan Co., Ltd.) for 5 min. Anti-mouse Gr-1 rat monoclonal antibody (diluted 1:200, MAB1037, R&D Systems, Inc., Minneapolis, MN) was applied to the sections overnight in a moisture chamber at 4ºC. The slides were then incubated using the MAX-PO kit (NICHIREI, Japan) for 30 min. The immunoreaction was visualized by the DAB reaction, and the sections were counterstained with hematoxylin.

8-OHdG staining was quantified by counting the stained nuclei (brown) and all nuclei (blue and brown) in 5 fields (240 × 320 μm) in each section for each animal (total of 3 animals). 4HNE and nitrotyrosine stainings were quantified by the ImageJ 1.40 g software. Brown color (the color of DAB staining) for 4HNE and red color (the color of AEC staining) for nitrotyrosine were extracted in 5 fields (50 × 50 μm) in each section for each animal (total of 3 animals), and the stained pixel numbers were calculated. The Gr-1 staining was quantified by counting the stained cells (brown) in 2 fields (1.9 × 1.4 mm) in each section for each animal (total of 3 animals).

223 **Preconditioning effect with either NO or H_2 breathing**

224 To investigate the preconditioning effect with either NO or H_2 breathing on the infarct
225 size, we placed anesthetized mice before the conventional 60-min occlusion of the left
226 anterior coronary artery; 1) with no preconditioning maneuver as a control, 2) under 3 times
227 5-min occlusion (ischemic preconditioning) in 5-min intervals, 3) under 3 times 5-min
228 exposure to 2% H_2 or 80 ppm NO (Fig. 7A). In all the procedure F_{IO_2} was fixed at 0.3.

229

230 **Statistics**

231 All data are presented as mean + SD. A one-way ANOVA with Tukey's *post hoc* test
232 was used for multiple-group comparisons for all groups. A two-way ANOVA with
233 replication was also used for comparison among the Control (antibiotics +), NO, H_2, and NO
234 plus H_2 groups, to determine the two-factor interaction between the effects of NO and H_2.
235 Additionally, a paired t-test was used to compare the results of echocardiography for mice
236 between before and after I-R.

237

238 **Results**

239 **Dose response curve of breathing H_2 gas concentration and infarct size**

240 Inhalation of 1 and 2% H_2 reduced murine myocardial I-R injury size and the effects did
241 not significantly differ from the effect of breathing 3% H_2 (Fig. 3), as was reported in rats

(10).

Inhaled NO and/or H_2 decreased the infarct size after I-R injury

The ratio of AAR to left ventricle did not differ among groups (data not shown). The infarct size/AAR was significantly higher in mice administered antibiotics. Breathing NO, H_2, NO plus H_2 significantly decreased the infarct size/AAR. Furthermore, the infarct size/AAR in the mice that inhaled NO plus H_2 was significantly lower than that in those that inhaled NO alone (Fig. 4A&B), whereas the antibiotics *i.m.* injection just before the I-R experiments did not show worsening in the infarct size (Fig. 4C), with the same AAR/LV ratio (data not shown), indicating no direct protective effect of antibiotics on the cardiac I-R injury. The interaction between NO and H_2 was significant (P=0.0052).

The effects of the inhaled gases on the LVEF

Movement of the cardiac anterior wall of control mice was reduced after I-R, but that of mice inhaling NO plus H_2 remained unchanged (Fig. 4D&E). The LVEF after I-R in mice breathing NO plus H_2 was significantly improved as compared with that of control mice (Fig.4E). The interaction between NO and H_2 was not significant (P=0.4467).

Immunohistochemical staining with antibodies against 8-hydroxy-2'-deoxyguanosine

(Fig. 5A&B)

The number of nuclei, calculated as a percentage of the total number of nuclei, that showed positive staining for 8-OHdG, was significantly decreased in mice that breathed NO, H_2, or NO plus H_2 as compared with that in the control mice. The number of positively stained nuclei in the mice that inhaled H_2 was significantly less than the value in mice that inhaled NO alone, but was not significantly different in mice inhaling NO plus H_2. The interaction between NO and H_2 was significant (P<0.0001).

Immunohistochemical staining with antibodies against 4-hydroxy-2-nonenal

Intense staining of the entire area for 4HNE was observed in the control groups (Fig. 5C). In the NO inhalation group, the stained area was reduced, and no staining was observed in the H_2 inhalation group or the NO plus H_2 inhalation group (Fig. 5D&E). The interaction between NO and H_2 was significant (P=0.0006).

Nitrotyrosine formation in the heart tissue

The nitrotyrosine-stained area expressed as a percentage of the total area in the region of interest was large in the control (both antibiotics – and +) groups and prominent in the NO inhalation group, whereas in the mice breathing NO plus H_2 the nitrotyrosine-staining was absent (Fig. 6A, B, C). The interaction between NO and H_2 was significant (P<0.0001).

280

281 **Neutrophil infiltration in the heart tissue**

282 The number of neutrophils in the region of interest in the slice was significantly
283 decreased in the mice that inhaled NO or NO plus H2 as compared with that in the control
284 animals (+)(Fig6 D, E). The interaction between NO and H_2 was significant (P=0.0225).

285

286 **Preconditioning effect with either NO or H_2 breathing**

287 As was observed and reported as the ischemic preconditioning effect before myocardial
288 I-R, preconditioning with NO breathing before the 60-min occlusion also had effects in
289 decreasing the infarct size, whereas H_2 breathing did not decrease the infarct size (Fig.7B).

290

291 **Discussion**

292 The new findings after murine myocardial I-R in this study include: 1) Enterobacterial
293 flora-derived H_2 slightly but significantly reduced myocardial infarct size; 2) NO plus H_2
294 breathing completely prevented the reduction of LVEF after I-R; 3) Either NO or H_2
295 breathing alone decreased infarct size. The reduction of infarct size was significantly greater
296 in mice that inhaled NO plus H_2 than in those breathing NO alone; 4) Staining for ROS
297 markers and neutrophils was reduced after NO and/or H_2 inhalation; 5) NO inhalation
298 enlarged the myocardial staining area for nitrotyrosine, whereas H_2 inhalation completely

eliminated the staining for nitrotyrosine; 6) Preconditioning with NO before I-R decreased the infarct area, while preconditioning with H_2 did not.; 7) Repetitive two-way ANOVA revealed a significant interaction between the effects of NO and H_2 on the infarct size, ROS and nitrotyrosine production, and neutrophil infiltration.

The finding that the presence of enterobacterial flora caused a slight but significant reduction in infarct size suggests loss of H_2-producing enterobacteria is a risk factor for massive infarction after I-R in the heart, and possibly in the brain, and may be clinically significant. It has also been reported in humans that the composition of the gut flora varies among individuals (21) from H_2-dominant production to methane-dominant. A number of probiotic supplements are reported to be cardioprotective, while their effect may not be related to H_2 production; on the other hand, the cardioprtective effect of dietary fiber (23) may be related to H_2 production. Poorly digestible fiber debris is transported to the distal intestine, where anaerobes ferment the debris, and produce not only H_2, but also methane, hydrogen sulfide, and so on. Therefore, it cannot be excluded that antibiotics also eliminated potential other cardioprotective gases. It would be interesting to study the specific protective effect of H_2 by inoculating wild-type bacteria producing H_2 and gene-manipulated bacteria unable to produce H_2 after clearing the enteric bacteria, similar to the strategy employed for demonstrating the protective role of H_2-producing *E. coli* against Concanavalin A-induced hepatitis (11).

318 Although the persistent, but low-dose production of H_2 by enteric bacteria may
319 contribute to reduction in the infarct size, or the dose-response relation for H_2 on the infarct
320 size may be very steep, it should be noted that enterobacterial flora-derived gases were not
321 sufficiently potent for cardioprotection after I-R injury, since inhalation of H_2 even in the
322 presence of enterobacterial flora significantly reduced the infarct size (Fig. 3).

323 Nagasaka *et al.* have shown that the cardioprotective effects of inhaled NO require the
324 presence of soluble guanylate cyclase-α_1, and the results of their study suggested that bone
325 marrow-derived cells are mediators of the ability of NO to attenuate cardiac I-R injury (18).
326 Therefore, it is possible that a primary role of NO is to inhibit recruitment of leukocytes from
327 blood stream to the heart. Their finding of the local effect of NO during myocardical I-R
328 injury is in line with our finding of reduced neutrophil infiltration observed following NO
329 inhalation, as well as of the *in vivo* preconditioning cardioprotective effect of NO-breathing
330 before the induction of I-R injury on the infarct size.

331 Ohsawa *et al.* showed in an *in vitro* study that H_2 gas can reduce hydroxyl radicals.
332 However, the reaction of H_2 with hydroxyl radicals is controversial. Since the half-life of
333 hydroxyl radicals is short, 10^{-9} sec (24), and Wood and Gladwin noted that the hydroxyl
334 radical is too short-lived to react with H_2, and will react with several cellular components
335 before reacting with less abundant H_2 molecules (29). As compared to hydroxyl radicals, the
336 half-life of peroxynitrite is long, 0.05-1 sec (24), and peroxynitrite has a greater chance to

react with H_2 in areas of I-R injury. H_2 molecules are very diffusible and may reach ischemic tissues even before reperfusion (10). During ischemia and reperfusion, they will eliminate peroxynitrite (and possibly hydroxyl radicals), thereby suppressing inflammation and cellular apoptosis. Concerning the mechanism of the effects of H_2 gas, other possible sites of action of H_2 have been suggested, such as induction of hemoxygenase 1 (4) and heat shock protein (28). These possibilities need to be further investigated. These possibilities should further be investigated.

A comparison of the effects of inhaled NO with those of inhaled H_2 in our study revealed that they exerted equivalent cardioprotective effect, both in terms of improving the cardiac function and reducing the infarct size. The ROS markers, 8-OHdG and 4HNE, were similarly eliminated. A major difference between the effects of NO and H_2 breathing is the production of nitrotyrosine in the tissue affected by I-R injury in NO breathing. The increased amount of nitrotyrosine produced by inhaled NO suggests that some adverse effects on signal trafficking and cellular function may occur sooner or later by the RNS reactions with the tyrosine at the active site of vital enzymes and cellular components.

It should be noted that we used the maximal concentration of NO, 80 ppm, as had been reported to be effective in the protection of heart against I-R injury, and furthermore we placed the mice under the minimal contribution of enterobacterial flora-derived H_2 by administrating antibiotics with fasting. It is possible that less NO inhalation with more

356 enterobacterial flora-derived H_2 might potently protect heart from I-R injury, but even in that
357 case H_2 inhalation would minimize the potential risk caused by nitrotyrosine production.
358 Other potential methods to reduce peroxynitrite production while maintaining the
359 beneficial effects of inhaled NO could be to use a peroxynitrite decomposition catalyst (14,
360 27) or increase the cGMP concentration in the cells by using a soluble guanylyl cyclase
361 activator(22) or phosphodiesterase-5 inhibitor (16), since these enhancers of NO effects via
362 cGMP induction may enable reduction of the inhaled NO concentration, thereby decreasing
363 peroxynitrite formation and minimizing the adverse effects of NO inhalation.
364 One of the limitations of this *in vivo* study was that young male mice were used for this
365 study, while myocardial I-R injury typically occurs in older individuals with comorbid risk
366 factors such as diabetes and atherosclerosis; furthermore, many cardioprotective interventions
367 that are effective in younger animals are not effective in older animals or in the presence of
368 risk factors, a phenomenon that may be related to chronic oxidative stress (31). Therefore, it
369 will be important to verify our study findings in older mice, and in relation to enterobacterial
370 flora-derived H_2.
371 In conclusion, inhaled NO suppresses the inflammation in I-R tissues, and H_2 eliminates
372 the adverse by-products of NO exposure, peroxynitrite. Thus, our study supports the view
373 that NO and H_2 are suggestive partners that can be used as a mixture for breathing during
374 coronary interventions.
375

376

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380

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386 **Disclosures**

387 None of the authors has any relationship with industry and financial associations that might

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497 **Figure legends**

498 Fig. 1. Experimental procedure.

499 Myocardial ischemia was induced by transient occlusion of the left anterior descending

500 coronary artery. After 60 min of ischemia, the coronary artery was reperfused and the thorax

501 was closed. Commencing 5 minutes before reperfusion, 2% H_2, 80 ppm (=0.008%) NO, or

502 NO (80 ppm) and H_2 (2%) was added to inspiratory gas at FIO_2 0.3 exposure from 5 min

503 before reperfusion and during the initial 30 min of reperfusion. The mouse was sacrificed and

504 the heart was harvested and evaluated 24 hrs after reperfusion.

505

506 Fig. 2. Measurement of H_2 concentration in the expired air of mice

507 A: Mice were placed in a cage of 500ml. Expired air of mice was forced to flow at 50 ml/min

508 into a sampling bag for 10 min. B: The concentration of H_2 in expired air of mice, which

509 were given antibiotics and were treated with fasting, decreased under the limit of detection.

510

511 Fig. 3. Dose response curve of breathing H_2 gas concentration and infarct size.

512 The dose dependency of inhaled H_2 on infarct size in reference to the area at risk showed that

513 inhalation of 1 and 2% H_2 reduced murine myocardial I-R injury size and that the effects did

514 not significantly differ from the effect of breathing 3% H_2.

515

Fig. 4. Effects of the inhaled gas species on infarct size and heart function.

A: Representative cross sections after triphenyltetrazolium chloride staining in each group. B, top: The ratio of the area at risk (AAR) to the total area of the left ventricle (LV), AAR/LV, did not differ among the groups. B: The ratio of infarct size to AAR, infarct size/AAR, was significantly larger in mice given antibiotics. Breathing NO, H_2, and NO plus H_2 significantly decreased the infarct size/AAR. The infarct size/AAR in mice that inhaled NO plus H_2 was significantly lower than that of the mice that breathed NO only. C: To exclude the possible direct protective effects of antibiotics on the infarct size after I-R, the antibiotics were given *p.o.* as in the protocol (Fig.1) or injected intramuscularly (*i.m.*) just before the I-R experiments, and no worsening in the infarct size by *i.m.* injection has been observed, indicating no direct effect of antibiotics on the infarct size. D: Typical echocardiographic image at 24 hrs after I-R. E: LVEF of mice before and at 24 hrs after I-R. LVEF was significantly decreased at 24 hrs after I-R, except in mice that inhaled NO plus H_2. LVEF after I-R in the mice inhaling NO plus H_2 was significantly improved to that in the control mice, while that in the mice inhaling either NO or H_2 alone was significantly less than that in the control mice. * $P < 0.05$ differs *vs.* control. # $P<0.05$ differs *vs.* NO inhalation. Data are presented as mean + SD.

Fig. 5. Immunohistochemical staining for 8-hydroxy-2'-deoxyguanosine and

535 4-hydroxy-2-nonenal.

536 A: Nuclei stained blue with hematoxylin and stained brown by the DAB reaction using

537 primary antibody against 8-hydroxy-2'-deoxyguanosine. B: the number of

538 8-hydroxy-2'-deoxyguanosine-immunoreactive nuclei, expressed as a percentage of the total

539 number of nuclei in the region of interest in the slice, was significantly decreased in the mice

540 that inhaled NO, H_2, or NO plus H_2. C: Nuclei stained blue with hematoxylin and cells

541 stained brown by the DAB reaction using antibody against 4-hydroxy-2-nonenal. D:

542 Histogram of the brown color of each image extracted by color deconvolution. The tone level

543 of the brown color component of each pixel was presented on a scale of 0 (brown) to 255

544 (white). E: The 4-hydroxy-2-nonenal-immunoreactive area was expressed as a percentage of

545 the total area in the region of interest of the slice. Intense staining for 4-hydroxy-2-nonenal

546 was observed in the entire infarct area in control groups. Inhalation of NO reduced the stained

547 area as compared with that of control group. The stained area was eliminated in both the H_2

548 group and the H_2 plus NO group. Data are presented as mean + SD.

549

550 Fig. 6. Immunohistochemical staining for nitrotyrosine and neutrophils.

551 A: Nuclei stained blue with hematoxylin and cells stained red by the AEC reaction using

552 antibody against with nitrotyrosine. B: Histogram of the red color regions of each image

553 extracted by color deconvolution. The tone level of the red color component of each pixel

554 was presented on a scale of 0 (red) to 255 (white). C: The nitrotyrosine-immunoreactive area
555 (number of pixels of red color) was expressed as a percentage of the total area covered by the
556 cells (number of pixels covered by the cells) in each section. The
557 nitrotyrosine-immunoreactive area was significantly larger in the NO group than in other
558 groups. Nitrotyrosine-immunostaining was completely absent in both the H_2 group and NO
559 plus H_2 groups. D: Neutrophils stained brown by the DAB reaction after incubation with a
560 primary antibody against granulocyte receptor-1, and the nuclei stained blue with
561 hematoxylin. E: The number of neutrophils in the region of interest in the slice was
562 significantly decreased in the mice that inhaled NO or NO plus H_2 as compared with that in
563 the control animals (+).
564 Data are presented as mean + SD.
565
566 Fig.7 Preconditioning effect with either NO or H_2 breathing
567 A: Ischemic preconditioning effect as well as preconditioning effect with NO or H_2
568 breathing on infarction size after I-R injury was investigated. The coronary artery was
569 occluded or mice breathed NO or H_2 for 5-min in 5-min intervals 3 times before the
570 conventional 60-min occlusion of the left anterior coronary artery. B: Preconditioning with
571 NO breathing before the 60-min occlusion also had effects in decreasing the infarct size,
572 whereas preconditioning with H_2 breathing did not decrease the infarct size.

A

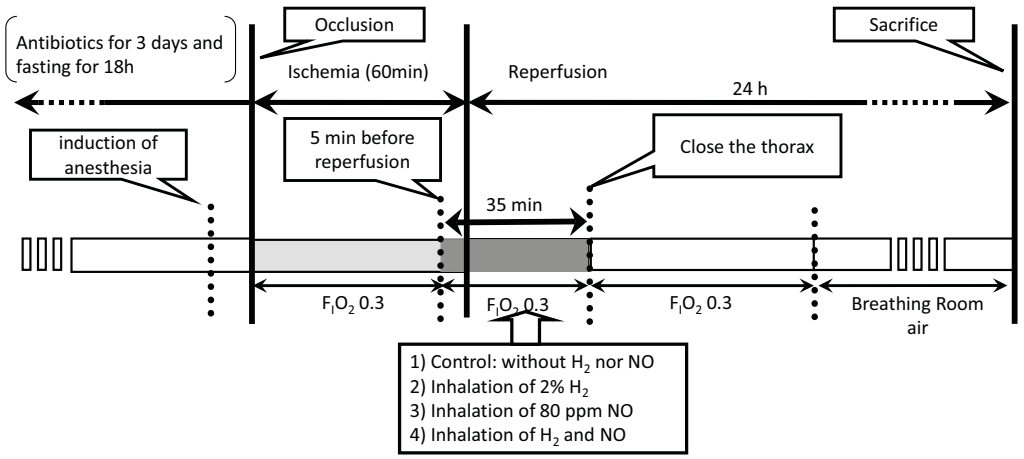

Antibiotics for 3 days and fasting for 18h
Occlusion
Ischemia (60min)
Reperfusion
Sacrifice
24 h
induction of anesthesia
5 min before reperfusion
Close the thorax
35 min
F_IO_2 0.3
F_IO_2 0.3
F_IO_2 0.3
Breathing Room air
1) Control: without H_2 nor NO
2) Inhalation of 2% H_2
3) Inhalation of 80 ppm NO
4) Inhalation of H_2 and NO

Fig. 1

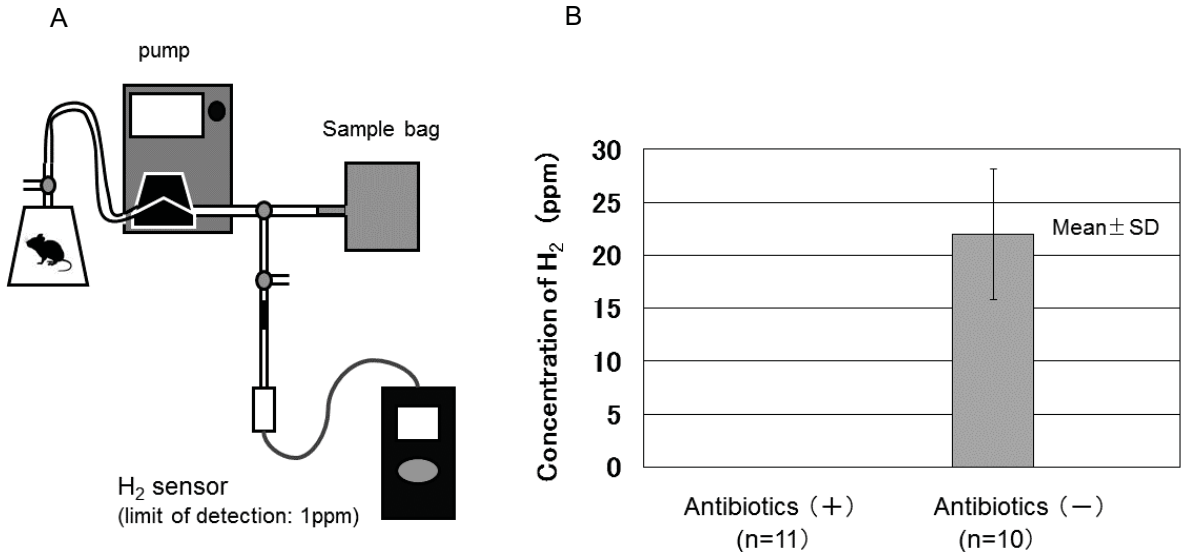

A
pump
Sample bag
H_2 sensor
(limit of detection: 1ppm)
B
Concentration of H_2 (ppm)
30
25
20
15
10
5
0
Mean±SD
Antibiotics（＋）
(n=11)
Antibiotics（－）
(n=10)

Fig. 2

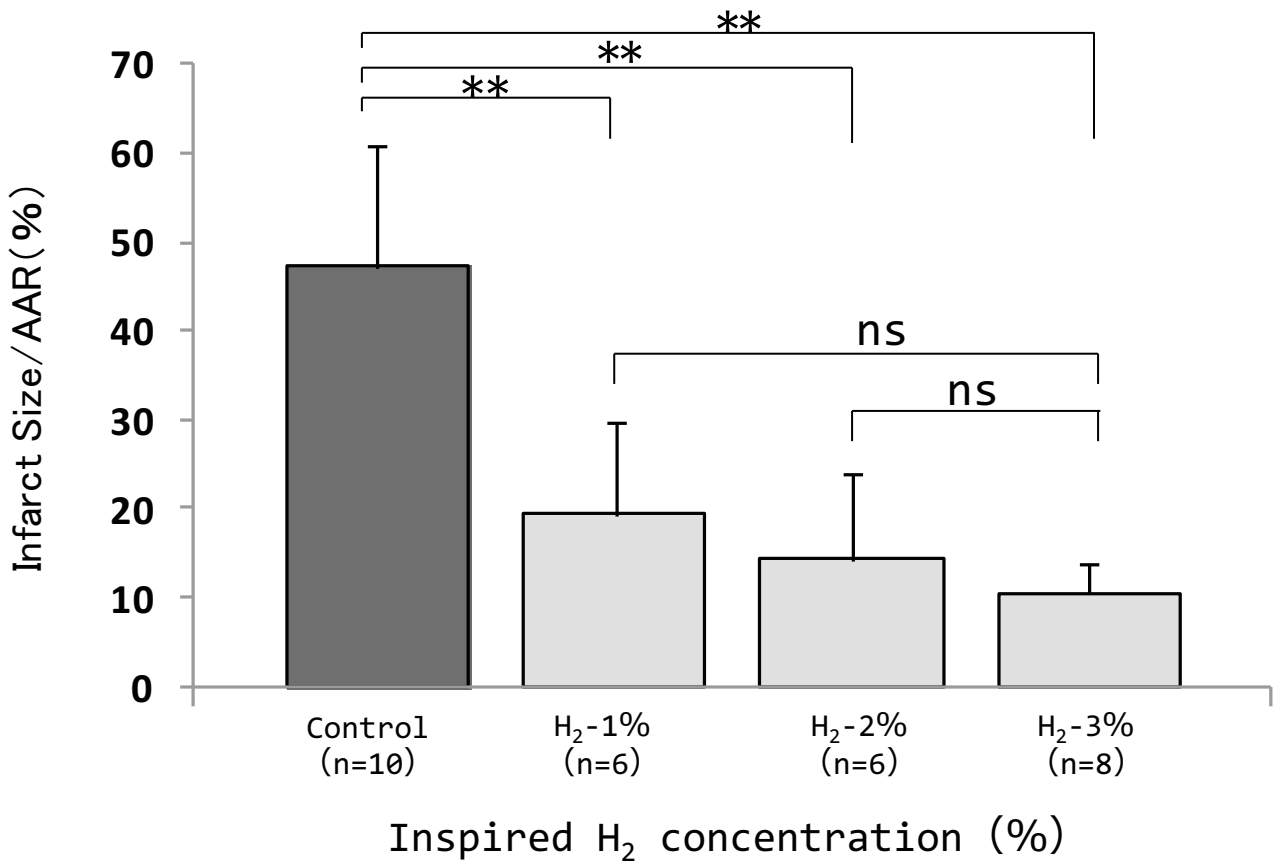

**
**
**
70
60
50
40
30
20
10
0
Infarct Size/AAR(%)
ns
ns
Control
(n=10)
H2-1%
(n=6)
H2-2%
(n=6)
H2-3%
(n=8)
Inspired H2 concentration (%)

Fig. 3

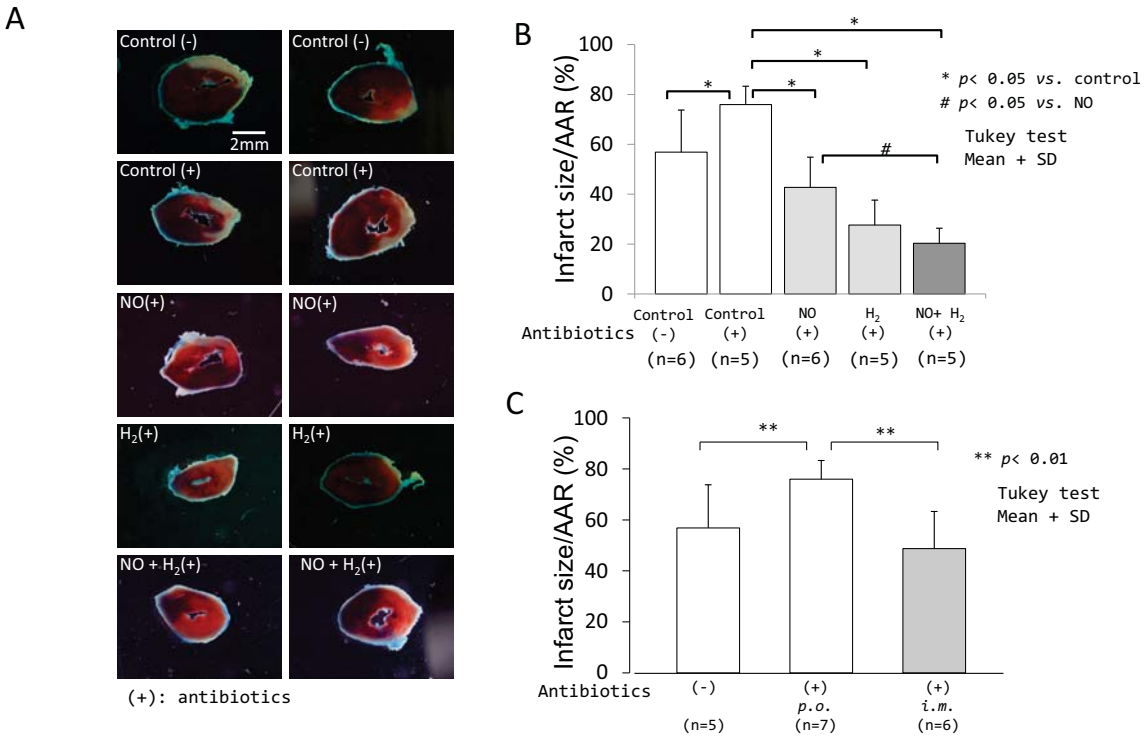

A
Control (-)
Control (-)
2mm
Control (+)
Control (+)
NO(+)
NO(+)
H2(+)
H2(+)
NO + H2(+)
NO + H2(+)
(+): antibiotics
B
Infarct size/AAR (%)
100
80
60
40
20
0
* p< 0.05 vs. control
# p< 0.05 vs. NO
Tukey test
Mean + SD
Control (-)
Control (+)
NO (+)
H2 (+)
NO+ H2 (+)
Antibiotics
(n=6) (n=5) (n=6) (n=5) (n=5)
C
Infarct size/AAR (%)
** p< 0.01
Tukey test
Mean + SD
(-)
(+) p.o.
(+) i.m.
Antibiotics
(n=5) (n=7) (n=6)
D
(Antibiotics)
Control (-)
Control (+)
NO (+)
H2 (+)
NO+H2 (+)
Before I-R
24h after I-R
E
LVEF (%)
N.S
n=5
Mean+SD
*p< 0.01
Paired t test
**p< 0.05
Tukey test
pre post
Control (-)
Control (+)
NO (+)
H2 (+)
NO+H2 (+)
(Antibiotics)

Fig. 4

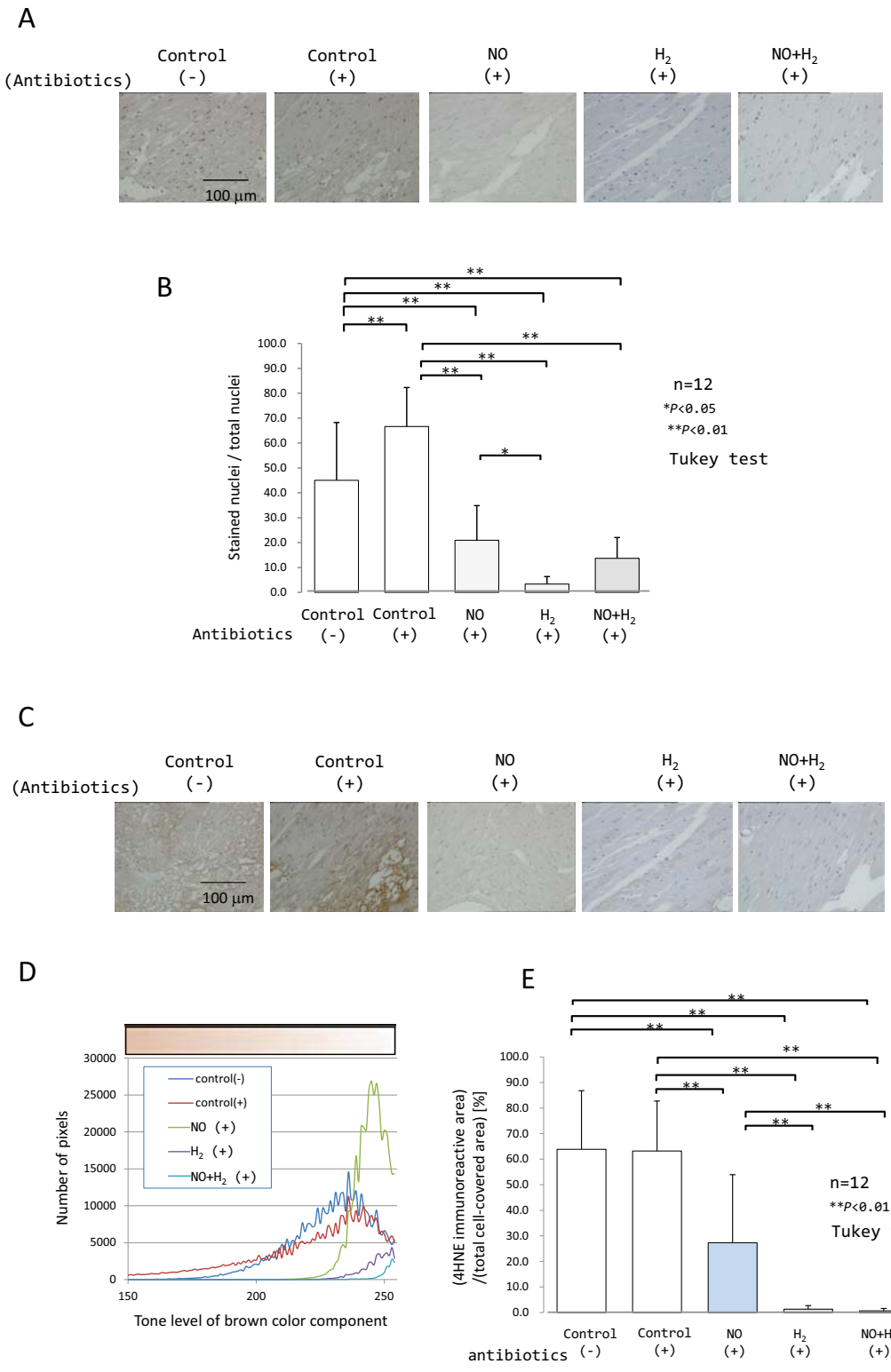

A
Control (-)
Control (+)
NO (+)
H2 (+)
NO+H2 (+)
(Antibiotics)
100 μm
B
Stained nuclei / total nuclei
Antibiotics
n=12
*P<0.05
**P<0.01
Tukey test
C
100 μm
D
Number of pixels
Tone level of brown color component
control(-)
control(+)
NO (+)
H2 (+)
NO+H2 (+)
E
(4HNE immunoreactive area) /(total cell-covered area) [%]
antibiotics
n=12
**P<0.01
Tukey test

Fig. 5

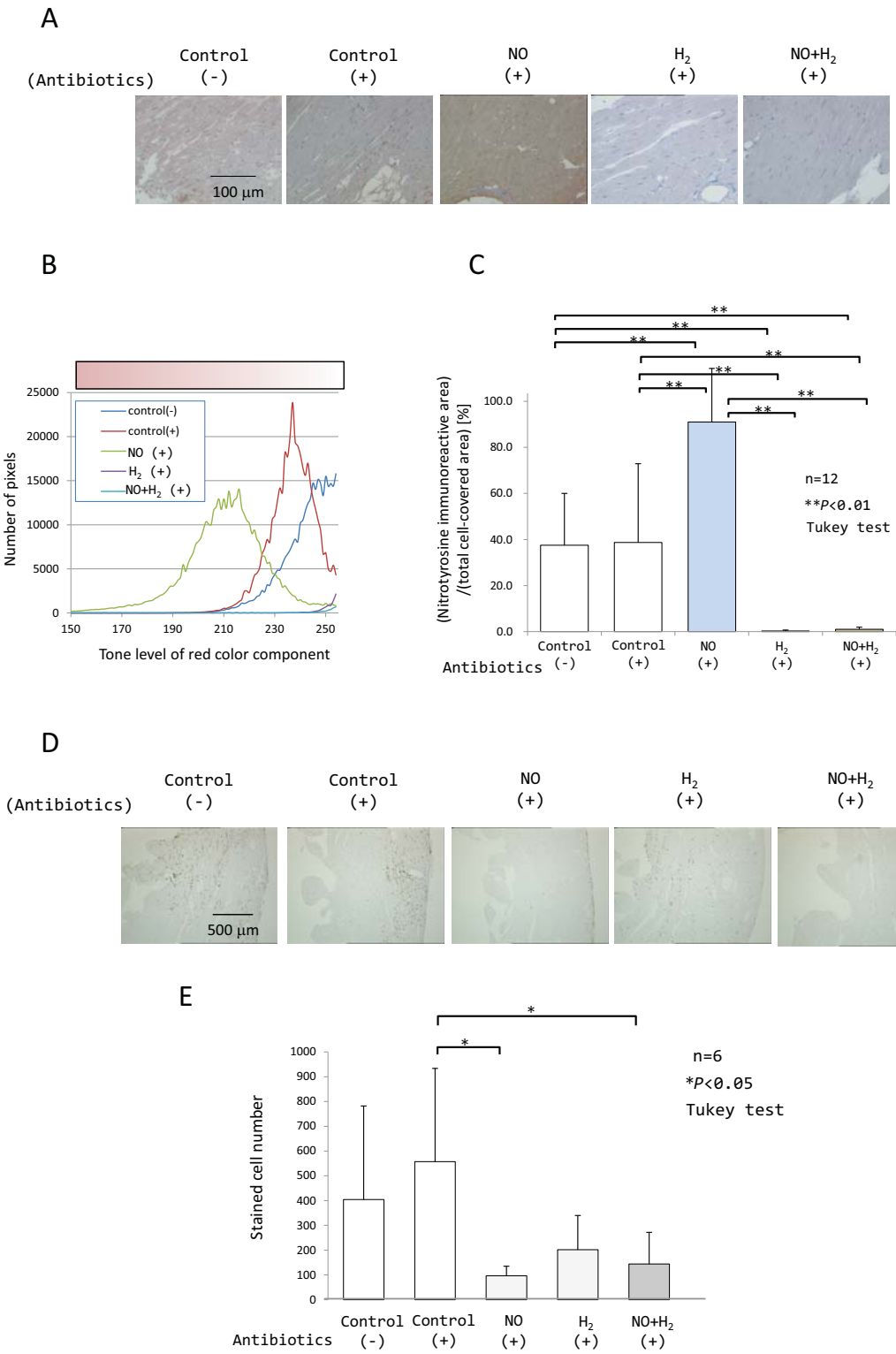
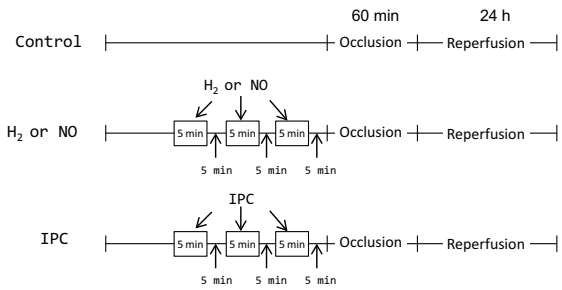

A
(Antibiotics)
Control (-)
Control (+)
NO (+)
H2 (+)
NO+H2 (+)
100 μm
B
Number of pixels
Tone level of red color component
control(-)
control(+)
NO (+)
H2 (+)
NO+H2 (+)
C
(Nitrotyrosine immunoreactive area) /(total cell-covered area) [%]
n=12
**P<0.01
Tukey test
Antibiotics
D
(Antibiotics)
500 μm
E
Stained cell number
n=6
*P<0.05
Tukey test
Antibiotics

Fig. 6

A


60 min
24 h
Control
Occlusion
Reperfusion
H2 or NO
H2 or NO
5 min
5 min
5 min
Occlusion
Reperfusion
5 min
5 min
5 min
IPC
IPC
5 min
5 min
5 min
Occlusion
Reperfusion
5 min
5 min
5 min

B

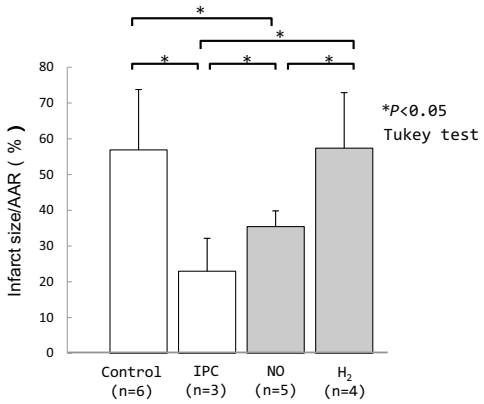

*
*
*
*
*
80
70
60
50
40
30
20
10
0
Infarct size/AAR (%)
*P<0.05
Tukey test
Control
(n=6)
IPC
(n=3)
NO
(n=5)
H2
(n=4)

Fig. 7