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Different effects of partial pressure in a high-pressure gaseous mixture of carbon monoxide and oxygen for rat heart preservation

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We maintained the function of an extracted rat heart after 24–48 h preservation in a high-pressure gaseous mixture of carbon monoxide (CO) and oxygen (O_2). Here, we assessed the effects of different partial pressures of hyperbaric CO and O_2 for 24–48 h at 4 °C on rat heart preservation and compared conditions including immersion in University of Wisconsin solution. Preserved hearts were transplanted into recipient rats via heterotopic cervical heart transplantation for *in vivo* evaluation and perfused using the Langendorff system for *ex vivo* evaluation. The survival rate of transplanted hearts was 100% at postoperative day 7 in the $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) group but only 33% in the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group. Langendorff system and histopathological analysis revealed that the left ventricular pressure of preserved hearts in the $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) group was better than the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm). We demonstrate that exposure of rat hearts to hyperbaric CO and O_2 is superior to the immersion method and that partial pressure of hyperbaric CO and O_2 is crucial to preservation.

Carbon monoxide (CO) is regarded as poisonous because of its high affinity for haemoglobin that causes rapid elevation of carboxyhemoglobin to toxic levels that compromise oxygen delivery to the tissues[1,2]. On the other hand, CO exerts vasoactive, antiproliferative, antioxidant, anti-inflammatory, and antiapoptotic effects and significantly contributes to cell protection[3]. Studies have suggested a beneficial effect of gaseous CO under pathophysiological conditions, such as organ transplantation, ischaemia reperfusion injury (IRI), inflammation, sepsis, or shock states[3,4]. However, hyperbaric O_2 treatment has been shown to damage lung function, potentially causing cellular dysfunction. Clinically, hyperbaric O_2 is only used in limited circumstances, such as after heart surgery, and extended periods of treatment are usually avoided. On the other hand, hyperbaric O_2 can reduce IRI that worsens crush injuries, induce the compartment syndrome, and cause skin flap and reattachment failures by inhibiting the adherence of neutrophils that release proteases and produce free radicals[5,6].

Currently, the preservation time for clinically extracted hearts prior to transplantation is 4–6 h[7]. Typically, preservation of a donor organ involves flushing and cooling the organ with a cold storage solution to remove blood components, then immersing it in a cold storage solution, in a process known as static cold storage (CS)[8,9]. CS solutions, such as University of Wisconsin (UW) and histidine–tryptophan–ketoglutarate solutions, have been developed to improve the quality of preserved organs and extend the acceptable preservation time limits for transplantation[10]. Recent studies have demonstrated the advantages of machine perfusion over CS preservation for kidney and liver transplantation[11,12]. Thus, the specific preservation method needs according to each organ characteristics and the original method should be developed for each organ.

We developed a high-pressure gas (HPG) preservation method using a mixture of CO and O_2 gases and succeeded in resuscitating extracted rat hearts following 48 h of preservation under conditions of $CO + O_2$ ($PCO:PO_2 = 4:3$ atm)[13]. It was also shown that rat heart function after 24 h of high-pressure preservation using a mixture of CO and O_2 gases was almost the same as control hearts[14]. Moreover, we reported successful transplantation of a rat limb following 7 days of preservation under conditions of $CO + O_2$ ($PCO:PO_2 = 4:3$ atm)[15]. We also showed significantly lower blood levels of creatine phosphokinase in recipients who received limbs

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preserved in a chamber containing CO and O_2 gases for 18 h after wrapped in saline-moistened gauze for 6 h compared with limbs only wrapped in saline-moistened gauze for 24 h[15]. It was also reported that hyperbaric CO and O_2 successfully preserved rat kidney grafts for 24 h by protecting tubular epithelial cells from apoptosis and inhibiting inflammation[16]. Surprisingly, no protective effect was observed under conditions of $CO + O_2$ ($PCO:PO_2 = 4:3$ atm) compared with preservation of a rat kidney for 24 h in a chamber filled with air (1 atm)[16]. Therefore, we selected a lower partial pressure mixed gas composed of $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) for kidney graft and found an improved graft survival[16]. In this way, the appropriate total and partial pressures differ for each organ, and the partial pressures of CO and O_2 for HPG remain unknown. This study aimed to evaluate the difference of partial pressure between CO and O_2 on HPG in rat hearts.

Results

Preservation methods in this study. HPG preservation requires specific conditions including pressure, humidity, medical gases, and gas phase. To verify the importance of these conditions for HPG preservation, hearts were extracted from rats and preserved for 48 h at 4 °C under eight different conditions: (1) chamber CO7-moisit group (Fig. 1a), hearts were hung inside a chamber filled with a mixture of CO and O_2 gas (7 atm; $PCO:PO_2 = 4:3$ atm) and a flask containing 50 mL of distilled water was placed inside to maintain humidity; (2) CO1-moisit group (Fig. 1b), hearts were hung inside a chamber filled with a mixture of CO and O_2 gas (1 atm; $PCO:PO_2 = 0.6:0.4$ atm) with a flask with 50 mL of distilled water; (3) Air7-moisit group (Fig. 1c), hearts were hung inside a chamber filled with 7 atm air with a flask with 50 mL of distilled water; (4) Air1-moisit group (Fig. 1d), hearts were hung inside a chamber filled with 1 atm air with a flask with 50 mL of distilled water; (5) CO7-dry group (Fig. 1e), hearts were hung inside a chamber filled with a mixture of CO and O_2 gas (7 atm; $PCO:PO_2 = 4:3$ atm) without distilled water; (6) UW-group (Fig. 1f), hearts were immersed in UW solution (Viaspan, Du Pont, Wilmington, DE, USA); (7) UW-CO7 group (Fig. 1g), hearts were immersed in UW solution then placed in a chamber filled with a mixture of CO and O_2 gas (7 atm; $PCO:PO_2 = 4:3$ atm); and (8) UW-CO group (Fig. 1h), hearts were immersed in UW solution into which CO had been dissolved according to the method described by Kohmoto *et al.*[17]. Briefly, UW solution was vigorously bubbled at 4 °C before use for 5 min with compressed 5% CO gas mixed in air. In order to maintain soluble CO in the UW solution, CO-bubbled and CO-equilibrated UW solution was kept in a tightly sealed container with a secured lid without an air layer. In all hearts, blood was removed using the Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2.2H_2O$, 1.2 mM $MgSO4.7H_2O$, 1.2 mM $NaH_2PO_4.2H_2O$, 25.0 mM $NaHCO_3$, and 11.1 mM glucose) prior to preservation. Preserved hearts within UW solution were reinfused with UW solution after blood removal. In addition, control group was harvested hearts from donor rats were immediately transplanted to the recipient rats without preservation.

Chamber humidity and changes of heart weight before and after 48 h of preservation using different methods. Chamber humidity and changes of heart weight before and after preservation in each group are shown in Table 1. The humidity in the CO7-dry group was significantly lower than that in the CO7-moist group ($61.0 \pm 1\%$ vs $96.3 \pm 1.06\%$, $P < 0.05$). In the other groups, the humidity was approximately 96% and was not significantly different compared with the CO7-moist group. The rate of change in the CO7-dry group was significantly higher than that of the CO7-moist group ($-19.2 \pm 5.43\%$ vs $-13.4 \pm 1.77\%$, $P < 0.05$). The heart weights in UW, UW-CO7, and UW-CO groups decreased (by approximately 15%) after preservation, and the decrease in these groups was greater than that in the CO7-moist group.

Revival and survival rates of transplanted hearts after 48 h of preservation using different methods. The revival and survival rates of transplanted hearts from each group are shown in Table 1. After the heterotopic cervical heart transplantation, the revival rate of post-transplanted hearts was 100% (12/12) in the CO7-moist and control groups, 80% (4/5) in CO7-dry group, 40% (2/5) in the Air7-moist group, and 83% (5/6) in the CO1-moist group but was 0% (0/5) in the Air1-moist group. All post-transplanted hearts from the three groups in which the extracted hearts were immersed in preservation solutions (UW, UW-CO7, and UW-CO groups) could not be resuscitated. The survival rate of the transplanted hearts on postoperative day 7 was 100% (8/8) in the control group and 75% (9/12) in the CO7-moist groups, but 0% in the other groups.

Revival and survival rates of transplanted hearts after 48 h of preservation under different partial pressures of CO-moist using HPG. The revival and survival rates of transplanted hearts after preservation under different partial pressures of CO and O_2 are shown in Table 2. After heterotopic cervical heart transplantation, the revival rate of post-transplanted hearts after perseveration with $CO + O_2$ ($PCO:PO_2 = 4:3$ atm) was 100% (12/12), whereas this was 67% (6/9) for $CO + O_2$ ($PCO:PO_2 = 3:4$ atm) and 33% (2/6) for $CO + O_2$ ($PCO:PO_2 = 3.5:3.5$ atm). The post-transplanted hearts preserved using CO ($PCO = 7$ atm) or O_2 ($PO_2 = 7$ atm) could not be resuscitated. The survival rate of post-transplanted hearts on postoperative day 7 after perseveration using $CO + O_2$ ($PCO:PO_2 = 4:3$ atm) was 75% (9/12), whereas this was 33% (3/9) using $CO + O_2$ ($PCO:PO_2 = 3:4$ atm).

The revival rate of post-transplanted hearts after perseveration was 67% (6/9) using $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm), 50% (4/8) using $CO + O_2$ ($PCO:PO_2 = 1.75:1.75$ atm), and 100% (12/12) using $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm). All post-transplanted hearts preserved using CO ($PCO = 3.5$ atm) or O_2 ($PO_2 = 3.5$ atm) could not be resuscitated. The survival rate of post-transplanted hearts at postoperative day 7 was 33% (3/9) after perseveration using $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) and 25% (2/8) using CO ($PCO:PO_2 = 1.75:1.75$ atm), whereas this was 100% (12/12) using $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm).
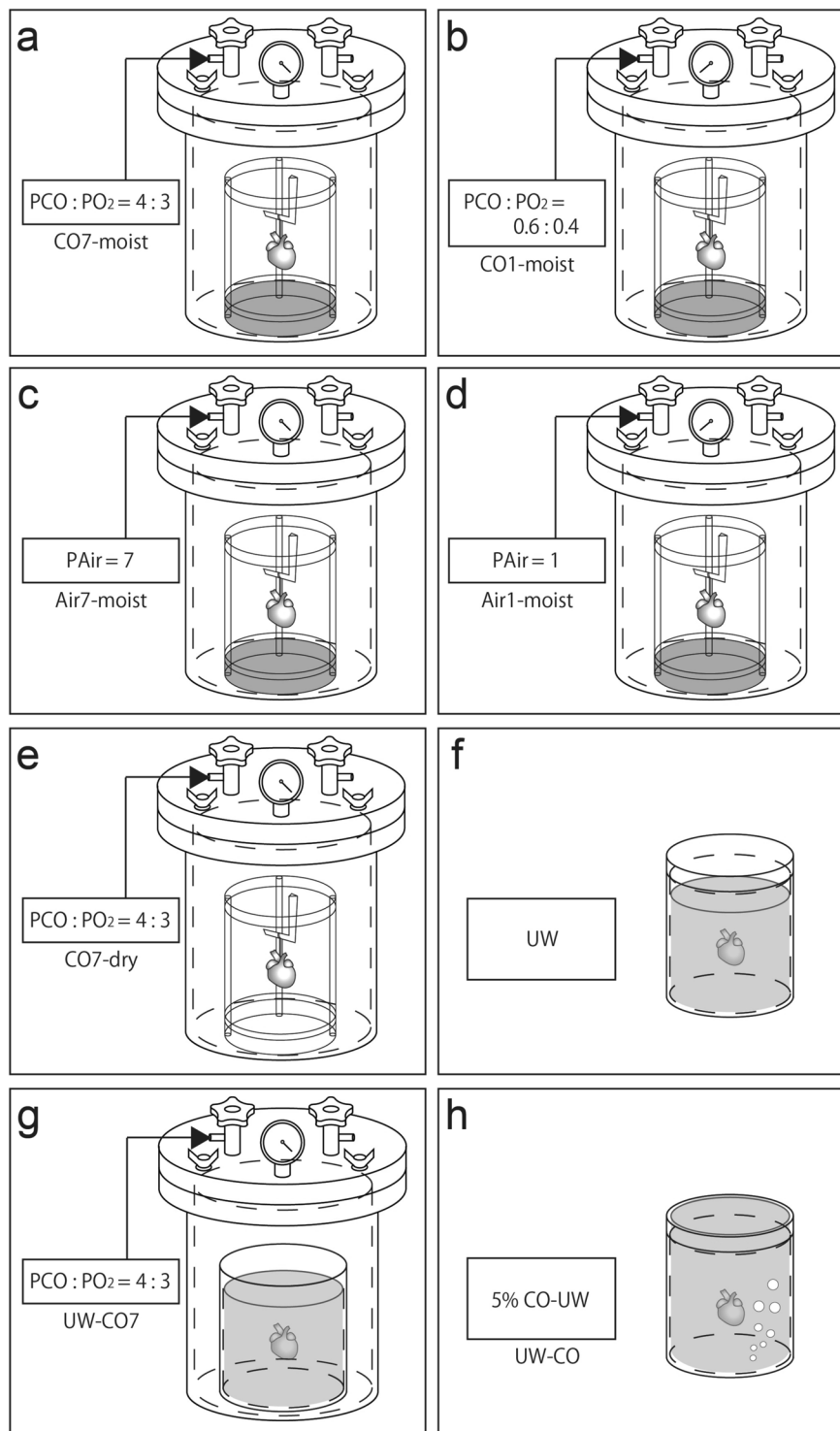
Figure 1. Conditions during rat heart extraction. (**a**) CO7-moist group, (**b**) CO1-moist group, (**c**) Air7-moist group, (**d**) Air1-moist group, (**e**) CO7-dry group, (**f**) UW-group, (**g**) UW-CO7 group, and (**h**) UW-CO group. CO, carbon monoxide; UW, University of Wisconsin solution.

The revival rate of post-transplanted hearts was 13% (1/8) after perseveration using $CO + O_2$ ($PCO:PO_2 = 0.6:0.4$ atm), 13% (1/8) using $CO + O_2$ ($PCO:PO_2 = 0.5:0.5$ atm), and 25% (2/8) using $CO + O_2$ ($PCO:PO_2 = 0.4:0.6$ atm). Post-transplanted hearts could not be resuscitated after preservation using CO ($PCO = 1$ atm) or O_2 ($PO_2 = 1$ atm).

Comparison of cardiac function after 24 h of preservation under conditions between $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) and $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) with heterotopic cervical heart

Group	Preservation method	Humidity in the chamber (%)	n	Heart weight			Revival rate (%)	Survival rate	
				before preservation (g)	after preservation (g)	rate of change (%)		at 1 hour (%)	on day 7 (%)
CO 7 -moist	Fig. 1a	96.3 ± 1.06^a	12	1.52 ± 0.27	1.31 ± 0.22^b	-13.40 ± 1.77^c	12/12 (100)	12/12 (100)	9/12 (75)
CO 1 -moist	Fig. 1b	96.3 ± 1.51	6	1.48 ± 0.24	1.28 ± 0.22	-13.85 ± 1.63	5/6 (83)	2/6 (33)	0/6 (0)
Air 7 -moist	Fig. 1c	96.4 ± 1.14	5	1.49 ± 0.25	1.30 ± 0.21	-13.20 ± 1.51	2/5 (40)	0/5 (0)	0/5 (0)
Air 1 -moist	Fig. 1d	96.1 ± 1.33	5	1.51 ± 0.24	1.33 ± 0.23	-11.87 ± 2.25	0/5 (0)	0/5 (0)	0/5 (0)
CO 7 -dry	Fig. 1e	61.0 ± 1.00^d	5	1.49 ± 0.31	1.19 ± 0.19^e	-19.20 ± 5.43^f	4/5 (80)	2/5 (40)	0/5 (0)
UW	Fig. 1f	–	5	1.42 ± 0.41	1.20 ± 0.35^g	-15.12 ± 2.11^h	0/5 (0)	0/5 (0)	0/5 (0)
UW -CO7	Fig. 1g	96.2 ± 1.10	6	1.44 ± 0.30	1.22 ± 0.25	-15.33 ± 2.06	0/6 (0)	0/6 (0)	0/6 (0)
UW -CO	Fig. 1h	—	5	1.45 ± 0.21	1.23 ± 0.16	-15.12 ± 2.77	0/5 (0)	0/5 (0)	0/5 (0)
Control							8/8 (100)	8/8 (100)	8/8 (100)

Table 1. Humidity in the chamber, change of heart weight before and after 48 h of preservation, and revival and survival rates of post-transplanted hearts using different preservation methods. Values are expressed as the mean SD. $P < 0.05$: a vs d, b vs e, g, c vs f, h. P-values were calculated by one-way ANOVA with post hoc Tukey's multiple comparison analysis.

Group	Partial pressure CO:O2 (atm)	n	Revival rate (%)	Survival rate on day 7 (%)
CO 7 -moist	7: 0	5	0/5 (0)	0/5 (0)
	4: 3	12	12/12 (100)	9/12 (75)
	3.5: 3.5	6	2/6 (33)	0/6 (0)
	3: 4	9	6/9 (67)	3/9 (33)
	0: 7	5	0/5 (0)	0/5 (0)
CO 3.5 -moist	3.5: 0	5	0/5 (0)	0/5 (0)
	2.0: 1.5	9	6/9 (67)	3/9 (33)
	1.75: 1.75	8	4/8 (50)	2/8 (25)
	1.5: 2.0	12	12/12 (100)	12/12 (100)
	0: 3.5	5	0/5 (0)	0/5 (0)
CO 1 -moist	1: 0	5	0/5 (0)	0/5 (0)
	0.6: 0.4	8	1/8 (13)	0/8 (0)
	0.5: 0.5	8	1/8 (13)	0/8 (0)
	0.4: 0.6	8	2/8 (25)	0/8 (0)
	0: 1	5	0/5 (0)	0/5 (0)

Table 2. Revival and survival rates of transplanted hearts after 48 h of preservation using different partial pressures of CO and O_2.

Transplantation. Measurement of the myocardial infarct area using 2,3,5-triphenyltetrazolium chloride (TTC) staining was significantly greater in the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group ($36.4\% \pm 5.96$) compared with the control ($8.4\% \pm 1.96$) and $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) ($10.8\% \pm 3.16$) groups ($P < 0.001$ for both). However, there was no significant difference between the control and $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) groups (Fig. 2a).

Light microscopy revealed that the myocardium in the $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) group maintained almost normal tissue structure and shape, with the myocardial fibres arranged in an orderly manner (Fig. 2b). However, the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group showed acute reperfusion injury characterised by haemorrhage (Fig. 2b).

Real-time reverse transcriptase PCR analyses revealed no changes in the expression of heme oxygenase-1 (HO-1) mRNA among the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm), $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm), and control groups (Fig. 2c). A large increase was noted in the mRNA expression of caspases 3, 8, and 9, and inducible nitric oxide synthase (iNOS), and nuclear factor-kappa B (NF-κB) in the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group but not in the control and $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) groups (Fig. 2c). Expression of tumour necrosis factor-alpha (TNF-α) significantly increased in the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) and $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) groups but not in the control group. Expression of interleukin-1 beta (IL-1β) was higher in the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group compared with the control and $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) groups (Fig. 2c).

Comparison of cardiac function after 6, 12, 18, 24, and 48 h of preservation under conditions between $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) and $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) using the Langendorff system. The results from the Langendorff system are shown in Fig. 3. After 48 h of preservation, hearts could beat in the $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) group but not in the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group. There was no significant difference of left ventricular pressure (LVP) after 6,
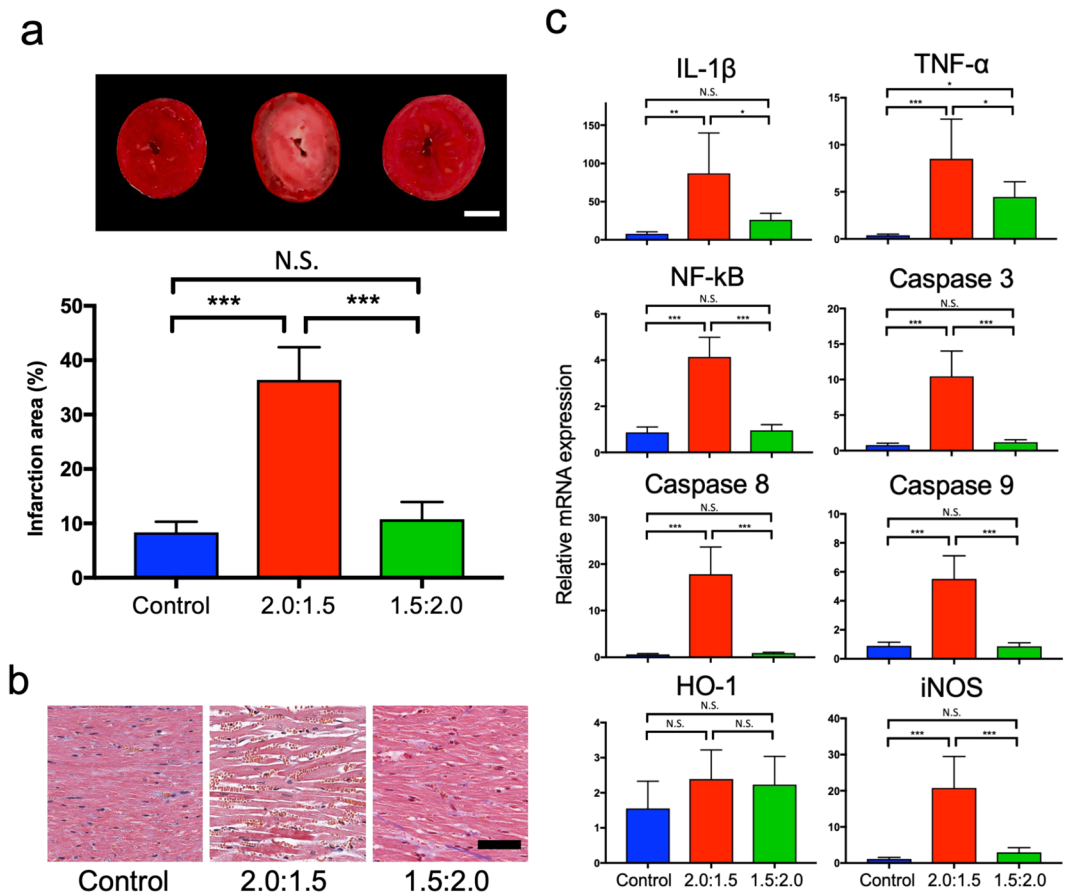
Figure 2. Cardiac function after 24 h of preservation under conditions between $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) and $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) using heterotopic cervical heart transplantation. (**a**) Representative mid-myocardial cross sections of TTC-stained hearts for the three groups (n = 6 for each group). Red-stained areas indicate viable tissue, and white areas indicate infarct tissue. White bar represents 500 μm. Myocardial infarction areas in the hearts from the three groups were quantified. $P < 0.05$ was considered statistically significant. N.S., not significant. ***$P < 0.001$. (**b**) Masson's trichrome staining in the hearts from the three groups. Black bar represents 50 μm. (**c**) mRNA expression levels measured by quantitative RT-PCR in grafted hearts (n = 6 for each group). N.S., not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. P-values were calculated by one-way ANOVA with post hoc Tukey's multiple comparison analysis.

12, and 18 h of preservation between the two groups, whereas LVP after 24 h of preservation was significantly higher in the $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) group compared with the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group. Heart rate after 6, 12, 18, and 24 h of preservation did not difference in the two groups. The ventricular pressure contractility (dP/dt) max after 6, 12, and 18 h of preservation did not differ between the two groups, whereas the dP/dt max after 24 h of preservation was significantly higher in the $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) group compared with the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group. On the other hand, the dP/dt min after 6 h of preservation did not differ between the two groups, whereas the dP/dt min after 12, 18, and 24 h of preservation was significantly lower in the $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) group compared with the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group.

Discussion

HPG preservation methods are useful to maintain organ function. There have been some reports about the optimum gas combination of O_2, CO, carbon dioxide, helium, and nitrogen for effective organ preservation. In the present study, we first demonstrated that exposure of rat hearts to hyperbaric CO and O_2 was better than using the immersion method, and IRI after preservation under conditions of $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) was lower than that of $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm).

Previous studies have demonstrated that the osmotic pressure of the solution is important for CS and machine perfusion[8–10]. UW solution has a high osmotic pressure to prevent tissue oedema and cell death and is suitable to preserve human hearts for 4–6 h[17]. Conversely, excessive drying was predicted during HPG preservation due to organ exposure to the gas phase. Therefore, we measured heart weights before and after preservation. Immersion of extracted hearts in UW solution for 48 h (UW, UW-CO7, and UW-CO groups in Table 1) reduced heart weights by approximately 15%, and the heart grafts could not be resuscitated. In the dry chamber with gases (humidity approximately 61%) (CO7-dry group), the weights of the preserved hearts were reduced by
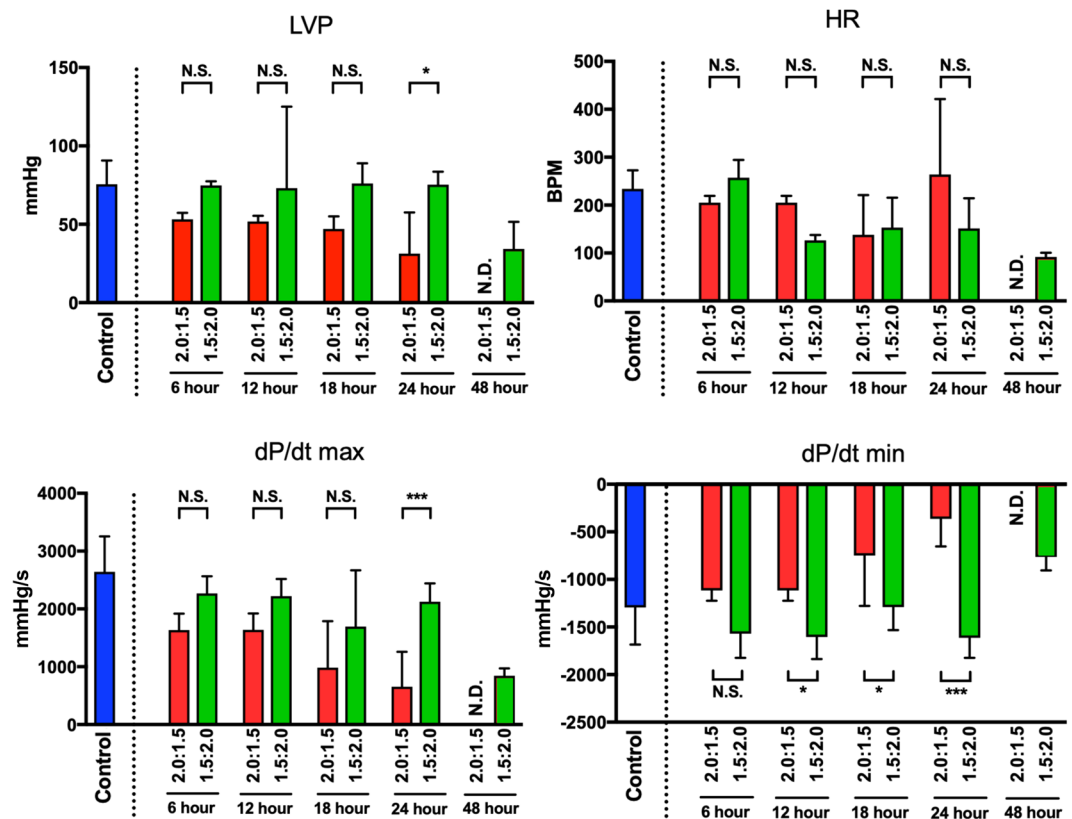
Figure 3. Cardiac function after 6, 12, 18, 24, and 48 h of preservation under conditions of $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) and $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) using the Langendorff system. Harvested hearts from control group rats were immediately perfused using the Langendorff system (n = 6). LVP, left ventricular pressure; HR, heart rate; dP/dt max and min, peak pressure increase and decrease; N.D., not detected; N.S., not significant, $*P < 0.05$, $***P < 0.001$. P-values were calculated by two-way ANOVA with post hoc Tukey's multiple comparison analysis.

approximately 19%, and function was not maintained. On the other hand, in the moist chamber with the same combination of gases (humidity approximately 96%) (CO 7-moist group), the preserved rat hearts were slightly dehydrated, and the weight decreased by only 13%, and 100% (12/12) of the heart grafts could be resuscitated. These results indicate that exposure of rat hearts to hyperbaric CO and O_2 is superior to the immersion method to preserve long-term cardiac function. While the dehydration worsens preservation state in HPG preservation method, therefore, preserved organs should be kept in humid conditions.

In the present study, we dissolved CO into UW solution according to a previous study and verified that heart grafts could not be resuscitated after 48 h of preservation (UW-CO group, 0/5 in Table 1)[18]. Furthermore, when the UW solution within the extracted hearts was under conditions of $CO + O_2$ ($PCO:PO_2 = 4:3$ atm), allowing CO and O_2 gases to dissolve into solution to a greater extent than at 1 atm, heart grafts could not be resuscitated after 48 h of preservation (UW-CO7 group: 0/6 in Table 1). These results suggest that CS using the immersion method of UW solution is limited to obtain the cell-protective effect of these gases on heart preservation. Heart grafts in the UW solution were under ambient pressure; however, resuscitation in UW solution under a pressure of 7 atm (UW-CO7 group) was not better than that under 1 atm (UW-CO group). These results indicated that the pressure itself may not improve storage conditions. Moreover, the survival rates of heart grafts in gases less than 7 atm (CO7-moisit group) was improved compared with those under 1 atm (CO1-moisit group) (Table 2). Henry's Law states that the amount of gas that is dissolved in a preserved organ under a pressure of 7 atm increases seven times more than that under 1 atm. It is thought that the different solubilities of solutions used for preserving hearts under pressure could be one of the reasons to extend preservation time on rat heart due to enhanced protective effects of CO and O_2. Conversely, we have previously described the preservation of rat kidney for 24 h and found no protective effects under conditions of $CO + O_2$ ($PCO:PO_2 = 4:3$ atm, 7ATA), whereas the use of $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm, 3.5ATA) improved the survival rate of the kidney graft[16]. This study also showed that the survival rates at postoperative day 7 using 3.5ATA ($PCO:PO_2 = 2.0:1.5$ atm; 12/12: 100%) were better than those using 7ATA ($PCO:PO_2 = 4:3$ atm; 9/12: 75%) on preserved rat hearts for 48 h with HPG preservation (Table 2). It is well known that high pressure causes organ damage because decompression injury and excessive CO and O_2 are highly toxic to preserved organs. Therefore, the use of the appropriate pressure is important for HPG preservation methods.

It was also found that either CO or O_2 alone could not preserve rat hearts for 48 h under high-pressure CO ($PCO = 7$ atm), O_2 ($PO_2 = 7$ atm), CO ($PCO = 3.5$ atm), O_2 ($PO_2 = 3.5$ atm), CO ($PCO = 1$ atm), and O_2 ($PO_2 = 1$ atm) groups

(Table 2). In regard to this combining (partial pressure) of CO and O2, surprisingly, in the present study, $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) reduced infarct area after 24 h preservation and improved the survival rate of heart grafts after 48 h preservation compared with $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm). Furthermore, the mRNA expression of the proinflammatory cytokines TNF-α and IL-1β was also significantly higher in the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group than in the $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) group (Fig. 2). These cytokines could stimulate NF-κB, a key transcription factor in the regulation of IRI, resulting in increased expression of apoptosis-related proteins caspase 3, 8, and 9. The increase of iNOS, a marker of oxidative stress, in the $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group may be also implicated in the pathophysiology of heart IRI. These results indicated that the effects of CO, such as anti-inflammation and anti-apoptosis, depend on the ratio of CO and O_2. Optimising the ratio of these gases could reduce the side effects of oxidative stress and IRI. Further investigations to identify the molecules associated with CO and O_2 will help elucidate the optimal conditions for HPG preservation.

We confirmed differences in partial pressures of CO and O_2 using the Langendorff system as an *ex vivo* evaluation (Fig. 3) and found that the results were similar to those using heterotopic cervical heart transplantation (Table 2). Moreover, the CO3.5-mosit ($PCO:PO_2 = 1.5:2.0$ atm) and CO7-moist ($PCO:PO_2 = 4:3$ atm) groups showed the highest survival rates and also showed similar trends in the analysis of the heterotopic cervical heart transplantation (Table 2) and Langendorff perfusion system (Supplementary Fig. S1). Further studies using orthotopic transplantation in large animals are required to better understand the detailed function of preserved hearts.

In conclusion, exposure of rat hearts to hyperbaric CO and O_2 without immersion in CS solution is effective to obtain the necessary cell-protective effects of gas for preservation. To date, many studies have reported the superiority of CS compared with machine perfusion; however, both methods were developed to expand the donor source, and these are not conflicting concepts. A protocol combining the advantages of CS and machine perfusion as well as other methods is required, and HPG using cell-protective gases may allow further expansion for organ preservation.

Methods

Animals.
An inbred line of LEW/SsN Slc rats (male, 10 weeks old; average weight, 230 g; range, 220–245 g; intact, n = 4; donors, n = 165; recipients, n = 165) was purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). Handling and care of the rats conformed to the National Institutes of Health guidelines for animal research, and all experimental protocols involving animals were approved by the Committee for Animal Care at Aichi Medical University (Permit No. 2016-11, 2017-46, 2018-70). All experiments involving animals were performed in accordance with the relevant guidelines and experimental protocols. Every effort was made to minimise animal suffering.

HPG preservation method.
For the HPG preservation method, we developed a chamber that could withstand high pressure (Nakamura Iron Works Co., Ltd. Tokyo, Japan) (Fig. 4). To prepare for organ preservation, the inner chamber was placed in distilled water to maintain humidity and then cooled to 4 °C. Extracted rat heart suspended in a plastic cylinder was placed in the chamber, and the lid was closed using four bolts. The chamber was filled with preservation gases. The inner chamber gas temperature was initially increased temporarily (peak 16.6 °C ± 0.8 °C) and the temperature was then decreased over approximately 80 s by placing the chamber in a fridge at 4 °C. Using this temperature alteration, the inner cardiac cavity temperature was increased by approximately 1.98 °C ± 0.53 °C (Supplementary Fig. S2). After preservation, the preserved heart was removed, and the gases were released from the chamber.

Evaluation of preserved rat hearts by heterotopic cervical heart transplantation.
Heterotopic cervical heart transplantation was performed as described previously by Heron et al.[19]. Briefly, hearts were extracted from male, 10-week-old donor rats (n = 165) under deep anaesthesia using pentobarbital (50 mg/kg; Kyoritsu Seiyaku Corporation, Tokyo, Japan). Blood was removed using Krebs–Henseleit solution following aortic/pulmonary artery incision and further infusion. Male, 10-week-old recipient rats (n = 165) were deeply anaesthetised with pentobarbital (65 mg/kg body weight) and kept under anaesthesia using isoflurane (ISOFLU, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) in the inhalation anaesthesia apparatus (Univentor 400 Anesthesia Unit, Univentor Ltd., Zejtun, Malta). Preserved donor rat hearts were transplanted into the recipient's cervical region. The aortic arch and pulmonary artery of the donor heart were overlaid at the end of the common carotid artery and external jugular vein with the cuff of the recipients and then fixed to each cuff with a circular silk ligature. The skin was sutured after the heart rate stabilised. The heart rate was observed to be palpating in the neck, and post-transplant hearts were examined by electrocardiogram. Absence of a waveform for 15 min was defined as cardiac arrest.

Estimation of myocardial infarct size.
The size of the myocardial infarction in the three groups [control group, n = 6; $CO + O_2$ ($PCO:PO_2 = 2.0:1.5$ atm) group, n = 6; and $CO + O_2$ ($PCO:PO_2 = 1.5:2.0$ atm) group, n = 6] was estimated by TTC staining. Briefly, after reperfusion, hearts were weighed and cut into 2-mm-thick slices vertical to the atrioventricular groove. The slices were stained by incubating in 2% TTC solution in phosphate buffer (0.1 M) at 37 °C for 15 min and then fixed in 4% paraformaldehyde solution. Infarct size (unstained by TTC) was measured by planimetry using Image Pro Plus 5.0 software (Media Cybernetics, Rockville, MD, USA) and was expressed as a percentage of the total heart.

Light microscopy.
Rat hearts (n = 18) from each group were fixed in 10% formalin (3.7% formaldehyde in water) for 3 h. The samples were then washed, dehydrated using an ethanol series, and embedded in paraffin. Serial 6 μm sections were cut using a microtome and evaluated by Masson's trichrome staining.
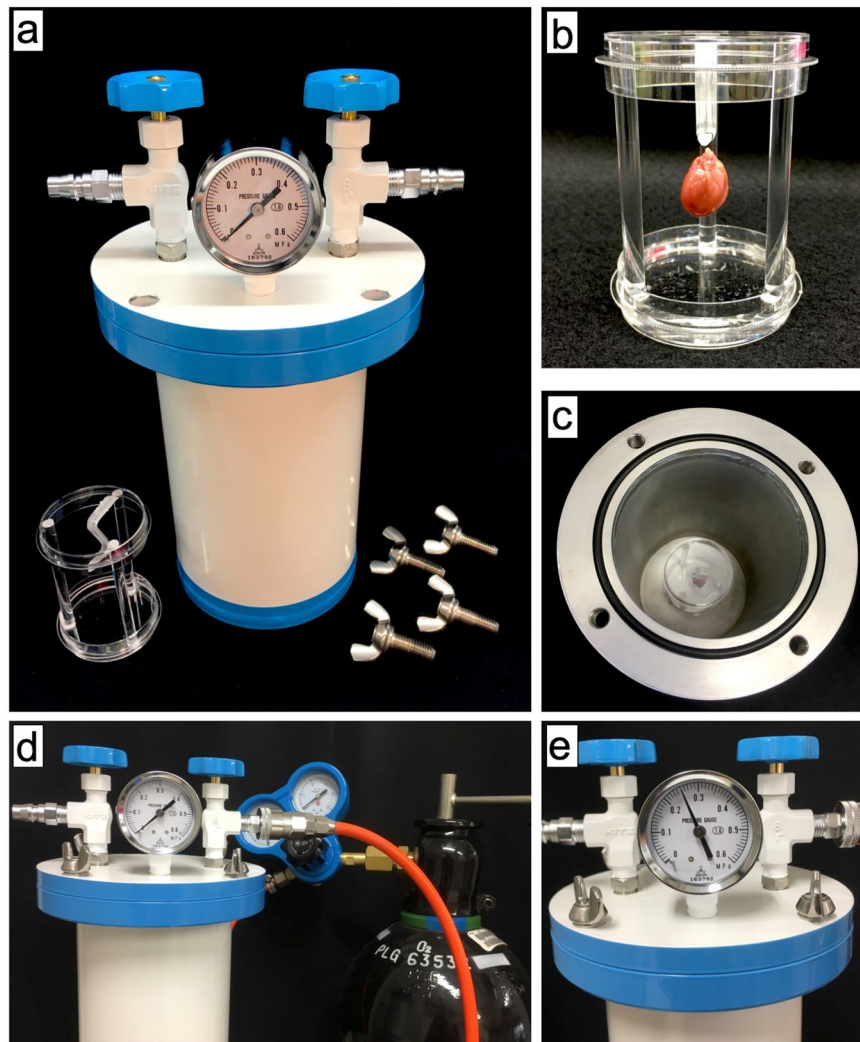
Figure 4. Photos of the HPG preservation method. (**a**) A 7 atm pressure-resistant chamber. (**b**) Extracted rat heart suspended in a plastic cylinder with water to maintain humidity. (**c**) Lumen of a chamber, into which a rat heart was placed. (**d**) Before filiation with CO and O_2. (**e**) After filiation with CO and O_2.

Gene expression analysis.   Hearts were obtained from each group at 90 min after transplantation [control group, n = 6; CO + O_2 (PCO:PO_2 = 2.0:1.5 atm) group, n = 6; and CO + O_2 (PCO:PO_2 = 1.5:2.0 atm) group, n = 6]. Total RNA was isolated from a sample of heart using a TRIzol RNA extraction kit (Invitrogen, CA, USA) according to the manufacturer's instructions. cDNA was prepared according to a standard protocol (high-capacity cDNA archive kit; PE Applied Biosystems, Foster City, CA, USA) and stored at 80 °C until analysed. Quantitative RT-PCR of the cDNA was performed using a validated SYBR Green gene expression assay along with the SYBR Premix Ex Taq II (TaKaRa Bio Inc., Ohtsu, Japan) to measure rat HO-1; caspases 3, 8, and 9; iNOS; NF-kB, TNF-α, IL-1β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All the primers used are listed in Table 3. Quantitative RT-PCR was performed in duplicate using a thermal cycler dice real-time system TP800 (TaKaRa), and data were analysed using the same system. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to quantify gene expression levels. Data for quantitative RT-PCR products were standardised to GAPDH, which was used as intact hearts. To confirm the specific amplification of the target genes, each gene product was further separated on a 1.5% agarose gel to detect single bands at the theoretical product sizes, and dissociation curves were analysed to detect single peaks.

Langendorff system.   Aortas were cannulated and extracted hearts (n = 66) were perfused by retrograde aortic perfusion with Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl$_2$•2H$_2$O, 1.2 mM MgSO4•7H$_2$O, 1.2 mM NaH$_2$PO$_4$•2H$_2$O, 25.0 mM NaHCO$_3$, and 11.1 mM glucose) was maintained at pH 7.4 by continuously bubbling with 95%:O_2/5%:CO_2, and the temperature was maintained at 37 °C. The flow was adjusted once the hearts were mounted on the Langendorff system within the first minute, and flow was maintained between 10 and 15 mL/g wet weight/min using a peristaltic pump (Radnoti LLC., CA, USA). Adequate perfusion was established in this constant flow system as indicated from the aortic perfusion pressure recording. Contractile parameters were measured by insertion of a fluid-filled latex balloon through the left atrium into the

Name	Accession Number	Direction	Sequence 5′ to 3′
IL-1β	NM_031512.2	forward	tgtgatgaaagacggcacac
		reverse	cttcttctttgggtattgtttgg
TNF-α	NM_012675.3	forward	tgtgcctcagcctcttctc
		reverse	gagcccatttgggaacttct
NF-kB	NM_145788.2	forward	ttccccaggaaaggctgt
		reverse	tggttaggaaaaggcttcca
Caspase 3	NM_012922.2	forward	ccgacttcctgtatgcttactcta
		reverse	catgacccgtcccttgaa
Caspase 8	NM_022277.1	forward	agagcctgagggaaagatgtc
		reverse	tcacatcatagttcacgccagt
Caspase 9	NM_031632.1	forward	cgtggtggtcatcctctctc
		reverse	gagcatccatctgtgccata
HO-1	NM_012580	forward	gtcaagcacagggtgacaga
		reverse	ctgcagctcctcaaacagc
iNOS	NM_012611	forward	cccagagtctctagacctcaaca
		reverse	catggtgaacacgttcttgg
GAPDH	M17701.1	forward	agctggtcatcaatgggaaa
		reverse	atttgatgttagcgggatcg

Table 3. Target gene names, accession numbers, and specific primer pair sequences.

left ventricle, connected to a pressure transducer (AD Instruments, UK), and the balloon volume was adjusted to give an end-diastolic pressure of <10 mmHg. Data were continuously recorded using a Power Lab 8 preamplifier/digitiser (AD Instruments, UK).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, CA, USA), and data are presented as the mean ± standard error of the mean. The one-way ANOVA and the two-way ANOVA with post hoc Tukey's multiple comparison analysis was performed to compare experimental groups. Statistical significance was set at $P < 0.05$.

Data Availability
All data generated during this study are included in this published article.

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Author Contributions

S.H., N.H. and M.N. conceived, designed and performed the experiments; S.H., N.H., M.N., K.F. and H.Y. analysed the data; N.H., M.I. and M.N. supplied the reagents, materials, and analysis tools; S.H., N.H., K.F., H.Y., M.I. and M.N. wrote the paper.

Additional Information

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