AMELIORATION OF RAT CARDIAC COLD ISCHEMIA/REPERFUSION INJURY WITH INHALED HYDROGEN, CARBON MONOXIDE, OR BOTH

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ABSTRACT

Background: Recent advances in novel medical gases, including hydrogen and carbon monoxide (CO), have demonstrated significant opportunities for therapeutic use. This study was designed to evaluate the effects of inhaled hydrogen, CO, or both on cold ischemia/reperfusion (I/R) injury of the myocardium.

Methods: Syngeneic heterotopic heart transplantation was performed in rats after 6 or 18 hours of cold ischemia in Celsior. Survival, morphology, apoptosis, and marker gene expression were assessed in the grafts after in vivo inhalation of hydrogen (1-3%), CO (50-250 ppm), both, or neither. Both donors and recipients were treated for 1 hour before and 1 hour after reperfusion.

Results: After 6 hours cold ischemia, inhalation of hydrogen (>2%) or CO (250 ppm) alone attenuated myocardial injury. Prolonged cold ischemia for 18 hours resulted in severe myocardial injury, and treatment with hydrogen or CO alone failed to demonstrate significant protection. Dual treatment with hydrogen and CO significantly attenuated I/R graft injury, reducing the infarcted area and decreasing in serum troponin I and CPK. Hydrogen treatment alone significantly reduced malondialdehyde levels and serum high-mobility group box-1 protein levels as compared with air-treated controls. In contrast, CO only marginally prevented lipid peroxidation, but suppressed I/R-induced mRNA upregulation for several proinflammatory mediators and reduced graft apoptosis.

Conclusions: Combined therapy with hydrogen and CO demonstrated enhanced therapeutic efficacy via both antioxidant and anti-inflammatory mechanisms, and may be potentially a clinically feasible approach for preventing cold I/R injury of the myocardium.

KEY WORDS:

carbon monoxide

cold storage

heart

hydrogen

ischemia/reperfusion

transplantation

ABBREVIATIONS

CAD: coronary artery disease

CPK: creatine phosphokinase

COHb: carboxyhemoglobin

HMGB1: high-mobility group box 1

HTx: heart transplantation

I/R: ischemia/reperfusionLV: left ventricular (LV)

MDA: malondyaldehyde

TTC: 2, 3, 5-triphenyltetrazolium chloride

TUNEL: Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling

INTRODUCTION

Ischemia/reperfusion (I/R) injury, obligate in the surgical procedure for HTx, is now recognized as a major determinant of primary graft dysfunction (1). I/R causes not only cardiomyocyte injury, but also endothelial cell damage, which in turn can promote subsequent development of graft coronary artery disease (CAD) (2-4).

Recently, Ohsawa et al. demonstrated that hydrogen (H_2) selectively reduces the levels of hydroxyl radicals (OH·) and peroxynitrite ($ONOO^-$) *in vitro* and also exerts potent antioxidant activity in an *in vivo* rat cerebral ischemia model (5). In addition, we and others have shown that CO inhalation at a low concentration prevents transplantation-associated I/R injury in several transplantation models (6-9). These actions of CO provide a wide range of effectiveness in protecting the transplanted grafts.

Based on the beneficial effects of hydrogen and CO demonstrated in previous studies, we hypothesized that hydrogen and/or CO may mitigate cardiac cold I/R injury, and may afford more potent therapeutic strategies for cardiac grafts with prolonged cold ischemia when administered together. The ultimate goal of this study is to establish a clinically-applicable strategy using medical gases for the treatment of I/R injury following heart transplantation. Our approach using hydrogen and CO could easily be incorporated into current interventional or surgical procedures without increasing their complexity (10). Our results may provide a new avenue for investigation regarding therapeutic gas use in patients.

MATERIALS AND METHODS

Animals

Inbred male Lewis (LEW, $RT1^l$) rats weighing 200-250 grams were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Animals were maintained in laminar flow cages in a specific pathogen-free animal facility at the University of Pittsburgh and fed a standard diet and water *ad libitum*. All procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the National Research Council's Guide for the Humane Care and Use of Laboratory Animals.

Heterotopic heart transplantation

Heterotopic HTx was performed as described previously (11). The excised grafts were prefused with and stored in Celsior (Sangstat Medical, Menlo Park, CA) at 4°C for 6 or 18 hours, and then transplanted into the abdomen of syngeneic recipient rats. The number of experiments per group (n) refers to the number of individual animals used for each end-point.

Gas exposure

Cylinders containing the following mixtures were purchased from Praxair (Dunbury, CT): hydrogen (1-3%) and balanced air; CO (50-250ppm) and balanced air; hydrogen (2%), CO (250ppm) and balanced air. Gas exposure was performed through an anesthetic gas vaporizer utilizing a facial mask during surgery. The treatment concentrations of hydrogen and CO were determined based on previous observations (5, 12-14). Both donors and recipients were treated 1 hour before and 1 hour after reperfusion. Air-treated transplant recipients and naïve control rats remained in normal atmospheric conditions for the duration of the study. Thus, the following animal groups were constructed: (i) naïve control rats anesthetized without surgery (NonTx/air), (ii) air-treated transplant recipients (HTx/air), (iii)

transplant recipients with hydrogen monotherapy (HTx/H_2), (iv) transplant recipients with CO monotherapy (HTx/CO), and (v) dual therapy transplant recipients (HTx/H_2 CO).

The recipients were sacrificed at 3 and 6 hours after reperfusion, and blood, heart graft and lung tissue samples were obtained. Half of each graft sample was snap-frozen and stored at -80°C until use. The remainder of each sample was fixed in 10% buffered formalin for histology. For the survival study, a separate set of recipient animals was followed until cessation of beating or for 7 days after HTx.

Measurement of myocardial injury enzymes and serum HMGB1

Serum troponin I and creatine phosphokinase (CPK) levels were measured using a Beckman autoanalyzer (Beckman Instruments, Fullerton, CA). Serum high-mobility group box 1 (HMGB1) was determined by ELISA (SHINO-TEST Co. Tokyo, Japan) according to manufacturer's protocol. The infarcted area of cardiac grafts can be visualized by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining as previously described (15).

Functional and macroscopic observations on the cardiac grafts

To assess cardiac graft function, we evaluated the gross morphology of the grafts, with their identities masked, and assigned a transplant score based on contractility (0; none, 1; moderate, 2; best), color (0; dark, 1; partially dark, 2; healthy), and turgor (0; hard, 1: soft) at 10 minutes and 3 hours post-reperfusion (16, 17). Graft failure was indicated by the cessation of cardiac contractions as monitored by abdominal palpation. Additionally, at 3 hours after reperfusion, echocardiography was performed using the Acuson Sequoia C256 system with a 13-MHz linear ultrasonic transducer (15L8; Acuson Co, Mountain View, CA) in a phased-array format. M-mode measurements on the left ventricular short-axis view (papillary muscle level) were performed.

Detection of infiltrating cells and apoptosis

Formalin-fixed, paraffin-embedded cardiac graft tissue and recipient lung tissue taken 6 hours after reperfusion were cut into 6μm sections, and stained with mouse anti-rat ED1 monoclonal antibody (Serotec, Raleigh, NC) and a naphthol AS-D chloroacetate esterase staining kit (Sigma Diagnostics, St. Louis, MO), respectively. Positively-stained cells in five random high-power fields (HPF) per section were counted with the samples' identities masked. The cardiac grafts were also investigated using using the ApopTag Peroxidase Kit (Intergen Co., Purchase, NY) for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay.

Western blot

Western blots were performed using mouse monoclonal antibodies for anti-cleaved caspase-3 (Cell Signaling, Boston, MA), and rabbit anti-human polyclonal antibody for human β-actin (Sigma) as described previously (13).

SYBR green real-time RT-PCR of cardiac grafts

At least two samples were obtained from each heart graft and analyzed for mRNA levels for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and inducible nitric oxide synthase (iNOS) by real-time reverse-transcriptase (RT)-PCR, as previously described (13, 14).

Assessment of graft oxidative injury

Tissue malondyaldehyde (MDA) level, an indicator of oxidative stress in cells and tissues, was assessed using the BIOXYTECH MDA-586 kit (OxisResearch, Portland, OR) according to the manufacturer's protocol.

Statistical analysis

The results are expressed as mean with standard deviation. Statistical analysis was performed using analysis of variance (ANOVA, repeated measurements). The EZAnalyze add-in for Microsoft Excel (www.ezanalyze.com) was used to perform the F-test with Bonferroni post hoc group comparisons where appropriate. A probability level of $p<0.05$ was considered to be statistically significant. Graft survivals were plotted using the Kaplan-Meier method, and the differences between groups were analyzed using the log-rank test.

RESULTS

Blood hydrogen and CO levels

Analysis using gas-chromatography (Biogas analyzer BAS-1000, Mitleben, Osaka, Japan) revealed that 1 hour inhalation of hydrogen at 1%, 2% and 3% resulted in elevation of the blood concentration of hydrogen from 134.5±28.4 ng/mL to 387.5±13.7, 582.8±56.8 and 768.0±106.6 ng/mL, respectively. Following 1 hour CO exposure at 50 ppm and 250 ppm, carboxyhemoglobin (COHb) levels in recipient animals determined by a OSM3 Hemoximeter (Radiometer, Copenhagen, Denmark) were 8.2±1.3% and 20.5±3.0 %, respectively (Figure 1A). These observations revealed inhaled gases were successfully delivered to the target organ via the circulation.

Both hydrogen and CO mitigated myocardial injury after 6 hours cold ischemia

Experiments to optimize a clinically-applicable effective and safe strategy of hydrogen delivery in the setting of HTx with 6 hours cold ischemia were designed. Inhalation of hydrogen over 2% significantly reduced serum levels of CPK and Tropinin I levels 3 hours after reperfusion, although 1% of hydrogen had a marginal effect (Figure1B). Similarly, consistent with previous observations (18), CO at 250ppm could prevent cardiac cold injury, while CO at 50ppm did not. These results revealed that single gas treatment with hydrogen over 2% or CO at 250ppm attenuated cardiac I/R injury after cold preservation for 6 hours.

Combined therapy with hydrogen and CO inhalation reduced myocardial injury after 18 hours cold ischemia

Prolongation of the safe cold ischemic time may enable expansion of the donor pool and would thus provide benefits for patients on the waiting lists. We designed the experiments to evaluate the efficacies of the gases on the grafts following cold preservation for 18 hours in Celsior. HTx after 18 hours of cold ischemia resulted in an ischemic area of >30% of the

graft. The ischemic area extended to the endocardium, disrupting the inner lining of the graft. While treatment with either hydrogen or CO alone failed to protect from myocardial injury, dual treatment with hydrogen and CO significantly attenuated graft injury, reducing the infarcted area to only 12% (Figure2A). Only dual treatment significantly reduced graft ischemic area, as identified by TTC staining. The serum concentrations of the myocardial injury markers, CPK and troponin I, were significantly elevated after air-treated HTx after 18 hours of cold storage in Celsior, correlating with the extent of morphological damage. Serum troponin I and CPK levels were significantly decreased when mixed gas therapy accompanied storage in Celsior solution (Figure2B and C).

Subsequent experiments utilized heart grafts with cold ischemia of 18 hours.

Dual treatment with hydrogen and CO had a protective effect on macroscopic morphology of grafts subjected to 18 hours ischemia

The transplant score, which quantifies the gross macroscopic appearance of the cardiac grafts, from rats receiving no treatment (air) 3 hours after storage in Celsior and reperfusion was 0.3±0.3. Hydrogen inhalation alone and combined gas therapy significantly improved the transplantation score to 2.9±1.3 and 3.1±0.7, respectively, whereas CO inhalation alone failed to prevent cardiac graft dysfunction (Figure2D). Prolonged I/R injury resulted in a deterioration of left ventricular (LV) function in the grafts. LV contraction in the grafts of rats exposed to air was hardly detected by echocardiography, whereas graft LV contraction was detected 3 hours after reperfusion in all animals receiving dual therapy with hydrogen and CO (Figure2E).

These early functional differences of the heterotopically transplanted heart grafts closely correlated with graft survival. Consistent with a previous report, no heart grafts survived >7 days when the grafts were stored in Celsior solution for 18 hours prior to transplantation if the recipients received no therapeutic gas (19). While only 10% of the recipients survived

>7 days after receiving CO treatment, dual therapy and hydrogen therapy alone significantly enhanced graft survival as compared with no treatment. Most strikingly, graft survival > 7 days was seen in 90% of the recipients receiving dual gas therapy (Figure2F). No animals treated with CO and/or hydrogen manifested neurological deficits during this 7-day follow-up period.

Graft infiltrating macrophages were decreased with dual therapy

Macrophage recruitment is a key feature of I/R injury and correlates with inflammatory events during the I/R process. In air-treated recipients of cardiac grafts with 18 hrs cold ischemia, macrophage infiltration of grafts was marked, as indicated by 40.7±12.4 ED1-positive macrophages/HPF 6 hours after reperfusion. Although monotherapy with hydrogen or CO did not significantly reduce macrophage recruitment, combined therapy with hydrogen and CO significantly decreased the number of ED1-positive macrophages to 18.5±5.5 ED-1 positive macrophages/HPF (Figure3A).

Combination therapy with CO and hydrogen reduced I/R-induced apoptosis

The TUNEL assay was performed to detect apoptosis in the heart grafts after 18 hours cold ischemia. I/R injury resulted in apoptosis of cardiomyocytes and vascular endothelial cells in control cardiac grafts, which was prominent by 6 hours after reperfusion (Figure3B). CO inhalation alone or in combination with hydrogen significantly reduced graft apoptosis. Western blot analysis demonstrated a close correlation between the number of TUNEL-positive cells and cleaved caspase-3 expression (Figure3C). There was a strong expression of cleaved caspase-3 in the grafts after treatment with air alone. Cleavage of caspase-3 was abrogated in groups receiving CO (with or without hydrogen), indicating anti-apoptotic effects of CO.

CO inhalation abolished upregulation mRNA for proinflammatory mediators

Prolonged (18 hours) cold I/R significantly upregulated several proinflammatory mediators (TNFα, IL-6, IL-1β and iNOS) within the grafts 3 hours after reperfusion as compared to naïve control rats. Although a trend towards a diminished upregulation of proinflammatory gene expression was seen in all gaseous treatment groups, a statistically significant decrease in graft mRNA levels for TNF-α, IL-6 and iNOS was reached only with CO treatment (alone and in combination with hydrogen) (Figure4A, B and D). Additionally, treatment with both gases was necessary to see a statistically significant decrease in IL-1β mRNA levels (Figure4C).

Hydrogen reduced graft lipid peroxidation

Tissue MDA levels, a marker of lipid peroxidation, in air-treated control recipients were significantly elevated from 0.43 μM/mg in control hearts of unoperated recipients to 0.83±0.05 μM/mg total protein 3 hours after HTx. Hydrogen treatment, either alone or in combination with CO, significantly attenuated lipid peroxidation, as indicated by reduced MDA levels (0.65±0.05 μM/mg). Monotherapy with CO did not prevent lipid peroxidation (Figure5A).

Hydrogen prevented HMGB-1 release

HMGB1-dependent inflammatory processes occur during I/R injury (20). Circulating HMGB1 levels were increased 3 hours after reperfusion in the air-control recipients of grafts. Therapy with inhaled hydrogen alone or in combination with CO significantly reduced serum HMGB1 levels (Figure5B). CO inhalation alone did not change serum HMGB1 levels 3 hours after reperfusion.

Dual therapy with hydrogen and CO significantly attenuated systemic inflammation

Prolonged cold I/R injury in the cardiac grafts may release inflammatory mediators, such as IL-1, and cause inflammation in remote organs, including the lungs (14, 17). While a few MPO-positive neutrophils were noted in naïve, unoperated rats, 6 hours after reperfusion there was a marked increase in MPO-positive cells in the lungs of graft recipients receiving no gas therapy. Additionally, the alveoli were more compressed in the air HTx group with loss of air space due to lung edema secondary to systemic inflammation (Figure6A). The combined therapy of hydrogen and CO significantly reduced the number of infiltrating neutrophils and decreased edema, suggesting that dual therapy decreased systemic inflammation associated with cardiac cold I/R injury (Figure6B).

DISCUSSION

This study provided evidence that inhaled hydrogen during transplant surgery, as well as inhaled CO, attenuated cold cardiac I/R injury. Also, we demonstrated that dual treatment with hydrogen and CO provided cumulative protection against cardiac cold I/R injury after prolonged ischemia, which was greater than treatment with either gas alone. Furthermore, combined gas therapy with hydrogen and CO mitigated remote organ inflammation secondary to graft cold I/R injury. Although CO alone had only marginal effects in mitigating oxidative injury to the grafts with severe cold I/R injury, hydrogen inhalation significantly reduced oxidant production, as evidenced by decreased levels of MDA. On the other hand, CO likely possesses direct anti-inflammatory effects, as shown by decreased upregulation of mRNA for several proinflammatory cytokines after CO treatment. This suggests that hydrogen and CO protect cardiac grafts from cold I/R injury through different mechanisms, warranting further study of the combined effects of CO and hydrogen.

Mitigation of I/R injury using this combination gas therapy could have enormous major beneficial impact on patient care. Although 1-year survival rates following clinical HTx have significantly improved to >80%, primary graft failure remains a major complication, with an incidence of about 10%, and contributes significantly to 30-day mortality (21). Additionally, survival declines at a linear rate after the first year posttransplantation, with a 5-year survival of only 70%, largely due to development of graft coronary artery disease (CAD) (*i.e.* transplant vasculopathy). Both graft CAD and primary graft failure are strongly correlated with the severity of graft damage from I/R injury at the time of transplant (3). Thus, therapy with inhaled hydrogen and/or CO could potentially improve survival in the early post-transplant period, improve long-term outcomes, and increase the use of marginal donors.

In the present study, hydrogen therapy exerted antioxidant effects as indicated by decreased lipid peroxidation in the transplanted hearts. The antioxidant properties of hydrogen have been demonstrated recently using models of several conditions, including ischemic heart disease (22), hepatic warm I/R injury (23), intestinal transplantation (14), or hypoxic brain injury (24). Because of the distinct, selective nature of hydroxyl and peroxynitrite inactivation, it is believed that hydrogen treatment does not eliminate superoxide anions (O_2^-) or hydrogen peroxide (H_2O_2) (5). Therefore, hydrogen therapy might spare the innate immune system (macrophages and neutrophils), which requires these reactive oxygen species (O_2^- and H_2O_2) to kill some types of phagocytosed bacteria. Hydrogen gas is physiologic and safe for humans. A low dose of hydrogen is continuously produced in the body by colonic bacteria and circulates in the blood under normal conditions (25). The safety of hydrogen is further demonstrated by its application for prevention of decompression sickness (26). CO is also continuously generated in the mammalian body through heme degradation and has antioxidant effects (27, 28).

Despite some similarities, there are several differences in the biological actions of hydrogen and CO. HMGB1 is a 30-kDa DNA-binding protein that displays proinflammatory, cytokine-like properties and plays a critical role in I/R injury as an early mediator of systemic inflammation and organ damage. Our data indicate that hydrogen inhalation significantly reduced HMGB1 release, while CO did not. In addition, our data clearly showed that CO potently inhibits the upregulation of inflammatory cytokine mRNAs, while hydrogen has only marginal effects. These data suggest that the molecular targets of hydrogen are different from those of CO.

Our data showed that hydrogen had a more profound anti-oxidant effect in the grafts that had undergone prolonged cold ischemia, as demonstrated by tissue lipid peroxidation. In contrast, CO had more marked anti-inflammatory and anti-apoptotic effects, as demonstrated by proinflammatory cytokine mRNA levels and TUNEL stain, respectively. Although we cannot clearly fully explain the discrepancy between the modest biochemical

differences seen with dual-gas treatment and the more striking impact of dual-gas treatment on graft survival, we hypothesize that mechanisms of action of hydrogen and CO are complementary to each other and that these complementary modes of action result in the superior graft survival after combined administration. The biochemical data reflect a candidate gene approach and, while not comprehensive, clearly show different pathways activated by each gas. A non-biased approach to identify biochemical changes is clearly worth pursuing in the future.

Alternatively, the absence of specific effects of the dual-gas therapy over either gas alone in the mechanistic analyses may be explained because the efficacy of each treatment may be easier to demonstrate by *in vitro* analyses of single parameters than by *in vivo* endpoints, including animal survival and graft function, which are likely the result of a complex biology influenced by many factors and their interactions. Therefore, it may be reasonable to observe the beneficial effects of the dual therapy with hydrogen and CO on graft survival, while other parameters, assessed by *in vitro* analyses are not exclusive to the dual-gas treatment. Nonetheless, the impact of dual-gas therapy with hydrogen and CO on graft survival is striking and is clearly the most important endpoint evaluated in this study.

At this stage, we chose to focus only on the combined effects of hydrogen and CO when treating both donor and recipient to maximize the beneficial effects. In future studies, it would be interesting to analyze the optimal timing and target (donor, recipient, or both) for each gas when administered jointly in the dual gas therapy.

In conclusion, our data demonstrate that the combination of hydrogen and/or CO is significantly effective in protecting against transplant-associated cold I/R injury. The combination of these two agents with differing, albeit overlapping, mechanisms of action, may have resulted in synergistic effects on graft survival. Mixed gas therapy with hydrogen and CO is a novel, safe and potent approach for preventing cardiac cold I/R injury. This study supports the clinical application of therapeutic medical gases and may justify a new avenue of investigation in therapeutic gas use in transplant patients.

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FIGURE LEGENDS

Figure 1: Dose-dependent effects of inhaled gas therapy in the rat heart transplantation model

A. Blood concentration of hydrogen (left panel) and blood COHb level (right panel) increased in a dose-dependent manner after inhalation of each gas for 1 hour (n=3).

B. Serum concentrations of CPK and Troponin I 3 hours after transplantation following 6 hours cold preservation in Celsior and treatment with hydrogen at different concentrations. (n=5 per group. F-test: P<0.05, *p<0.05 vs HTx air)

C. Serum concentrations of CPK and troponin I 3 hours after transplantation following 6 hours cold preservation in Celsior and treatment with inhaled CO at different concentrations. (n=5 per group. F-test: P<0.01, *p<0.05 vs HTx air)

Figure 2: The effects of inhaled gas therapy on oxidative injury of heterotopic cardiac grafts with 18 hours cold ischemia

A. The infarcted area of the grafts following 18 hours cold ischemia was visualized by TTC stain 3 hours after reperfusion. Representative slices of the grafts from each group are shown in the upper panel. (nonTx: naïve (unoperated) heart, HTx: heart transplant, n=5 per group. F-test: P<0.001, *p=0.012, HTx air vs HTx H_2/CO).

B. Serum troponin I levels at 3 hours after reperfusion. (n=10 for each group, F-test: P<0.001, *p=0.02 vs HTx air in Celsior solution)

C. Serum CPK levels 3 hours after reperfusion. (n=10 for each group, F-test: P<0.001, *p=0.14, HTx air vs HTx H_2; □p=0.004, HTx air vs HTx H_2/CO)

D. Transplant score to evaluate gross functional differences 10 minutes (gray bar) and 3 hours (black bar) after HTx. (n=6, 10 min, F-test: P=0.011, *p=0.02, HTx air vs HTx H_2/CO; 3 hours, **p=0.045, HTx air vs HTx H_2; $^{\delta}$p=0.004 HTx air vs HTx H_2/CO)

E. Representative M-mode echocardiography tracing of donor LV graft exposed to air (HTx air) or hydrogen and CO gases (HTx H_2/CO) with Celsior solution 3 hours after reperfusion. (n=3 for each group)

F. Graft survival by treatment group after 18 hours cold preservation in Celsior (n=10 per group). Results of Kaplan-Meier, log-rank test: p=0.0043, HTx air vs HTx H_2; p=0.0001, HTx air vs HTx H_2/CO; p=0.005, HTx CO vs HTx H_2/CO.

Figure 3: Detection of infiltrating macrophages and TUNEL-positive apoptotic cells in grafts following 18 hours cold ischemia in Celsior

A. Representative histopathological images of the heterotopic cardiac grafts stained for ED1 6 hours after reperfusion. The ED1 positive cells are red and are marked by arrows (original magnification, 400X). Histogram indicates the number of ED1-positive cells/HPF. (n=5, F-test: P=0.002, *p=0.049, HTx/air vs HTx H_2/CO)

B. Apoptosis in vascular endothelial cells (arrowheads) and cardiomyocytes (arrows) in the cardiac grafts stored for 18 hours in Celsior solution was assessed by TUNEL staining (original magnification, 400X). Numbers of apoptotic cells determined by TUNEL staining are shown in the right panel (n=5, F-test: P<0.001, *p=0.042, HTx air vs HTx CO; $^{\delta}$p=0.023, HTx air vs HTx H_2/CO).

C. Western blot analysis of cleaved caspase-3 in the grafts in recipients treated with air 6 hours after reperfusion. Representative pictures of Western blot from 3 independent experiments for each antibody are shown.

Figure 4: Quantitative RT-PCR for inflammatory mediators

Real-time RT-PCR for TNFα (A), IL-6 (B), IL-1β (C) and iNOS (D) mRNA in heterotopic cardiac grafts after cold storage in Celsior for 18 hours and reperfusion. (n=6, TNFα; F-test: P<0.001, *p=0.04, HTx air vs HTx CO; $^{\delta}$p=0.007, HTx air vs HTx H_2/CO, IL-6; F-test: P<0.001, *p=0.016, HTx air vs HTx CO; $^{\delta}$p=0.007, HTx air vs HTx H_2/CO, IL-1β; F-test: P<0.001, *p=0.03, HTx air vs HTx H_2/CO, iNOS; F-test: P<0.001, *p=0.011, HTx air vs HTx CO; $^{\delta}$p=0.03, HTx air vs HTx H_2/CO).

Figure 5: Graft MDA and serum HMGB1 levels

A. Graft MDA levels 3 hours after reperfusion following 18 hours cold ischemia in Celsior. (n=5, F-test: P<0.001, *p=0.002, HTx air vs HTx H_2; $^{\delta}$p=0.007, HTx air vs HTx H_2/CO).

B. Serum HMGB1 levels 3 hours after reperfusion. (n=5, F-test: P<0.001, *p=0.002, HTx air vs HTx H_2; $^{\delta}$p=0.026, HTx air vs HTx H_2/CO).

Figure 6: Recruitment of neutrophils in the recipient lungs after HTx

A. Representative images of recipient lung tissue stained for MPO-positive neutrophils 6 hours after reperfusion following 18 hours cold ischemia in Celsior. Arrows show the MPO-positive cells. (original magnification, 200X).

B. Number of MPO-positive cells per HPF (n=5, F-test: P<0.001, *p=0.04, HTx/air vs HTx H_2/CO).

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Figure 1

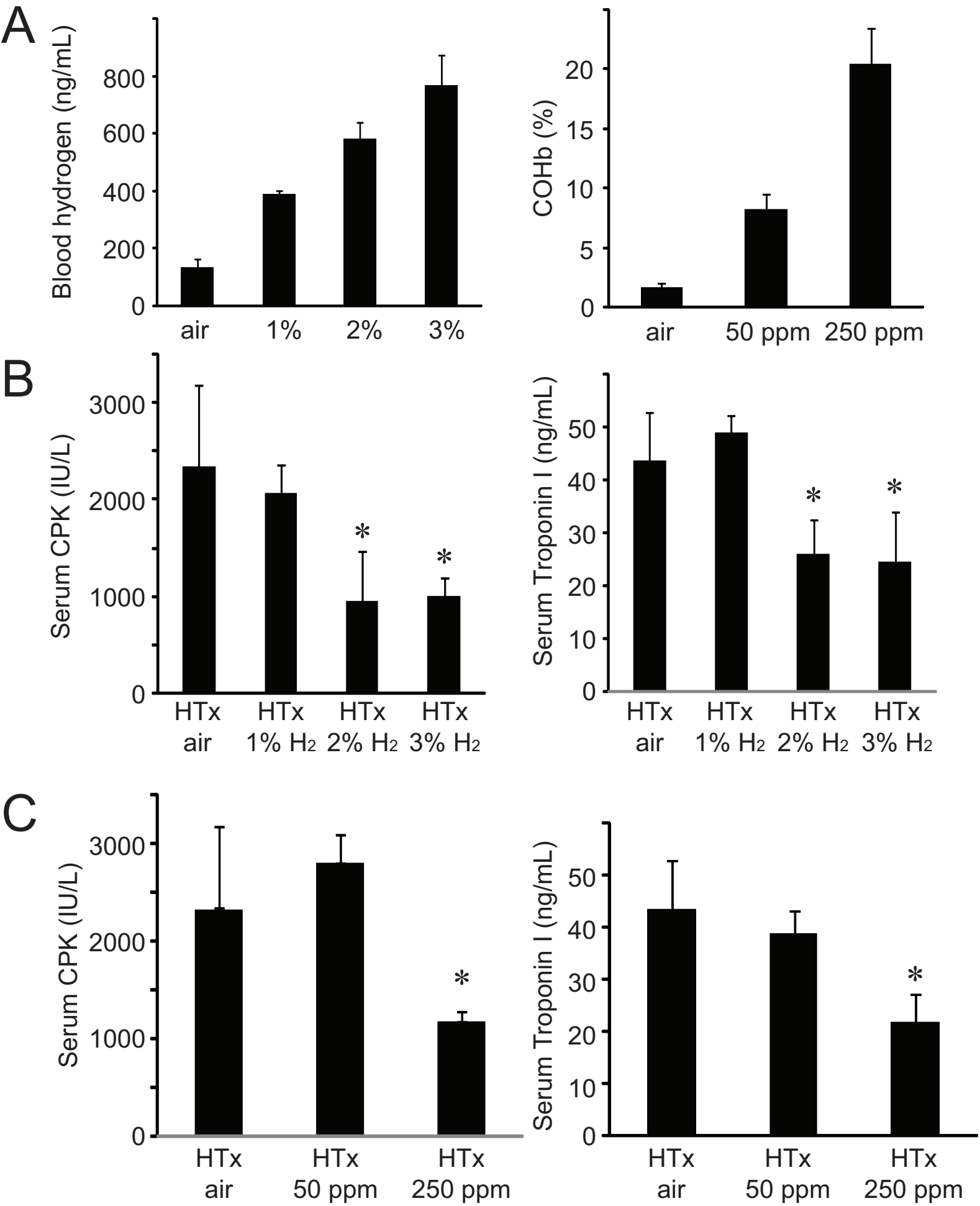

A
Blood hydrogen (ng/mL)
0
200
400
600
800
air
1%
2%
3%
COHb (%)
0
5
10
15
20
air
50 ppm
250 ppm
B
Serum CPK (IU/L)
0
1000
2000
3000
HTx air
HTx 1% H2
HTx 2% H2
HTx 3% H2
*
*
Serum Troponin I (ng/mL)
0
10
20
30
40
50
HTx air
HTx 1% H2
HTx 2% H2
HTx 3% H2
*
*
C
Serum CPK (IU/L)
0
1000
2000
3000
HTx air
HTx 50 ppm
HTx 250 ppm
*
Serum Troponin I (ng/mL)
0
10
20
30
40
50
HTx air
HTx 50 ppm
HTx 250 ppm
*

Figure 2ABC

Figure 2

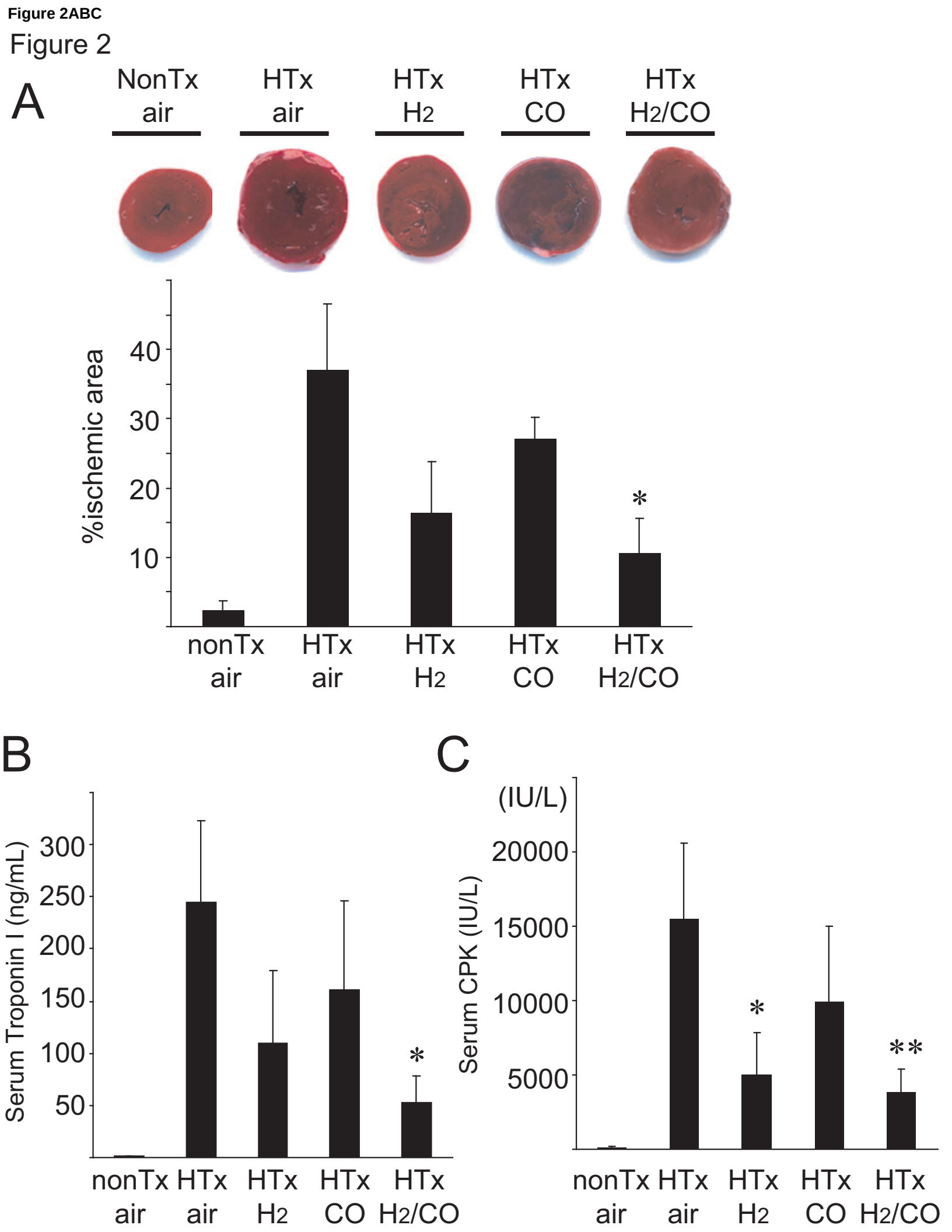
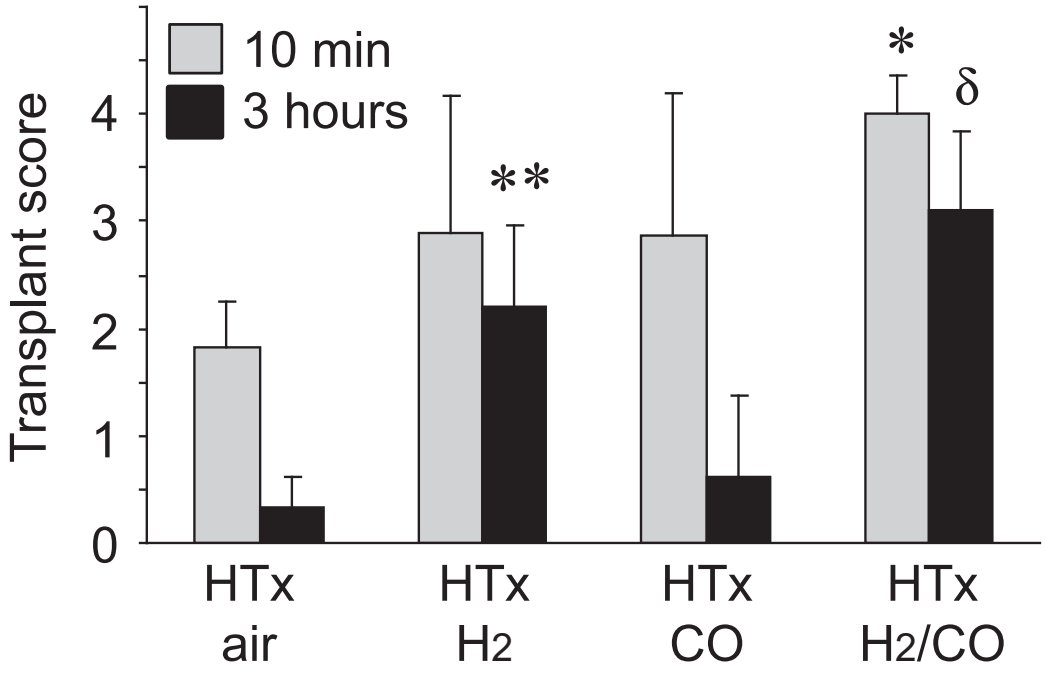
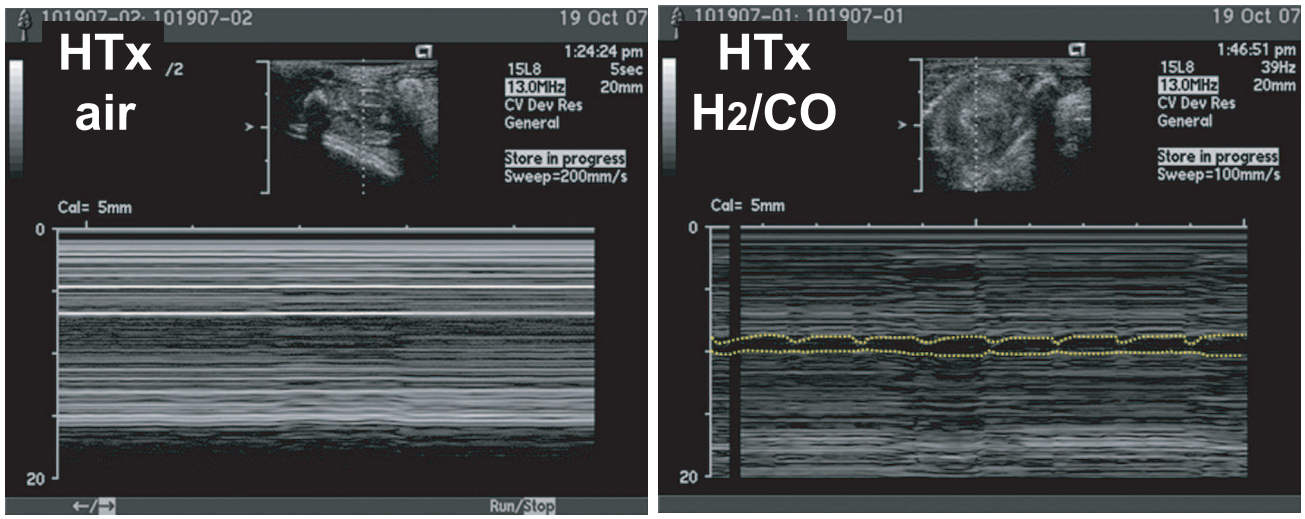


Figure 2

D


10 min
3 hours
Transplant score
0
1
2
3
4
**
*
δ
HTx air
HTx H2
HTx CO
HTx H2/CO

E


HTx air
19 Oct 07
1:24:24 pm
Store in progress
Sweep=200mm/s
Cal= 5mm
HTx H2/CO
19 Oct 07
1:46:51 pm
Store in progress
Sweep=100mm/s
Cal= 5mm

F

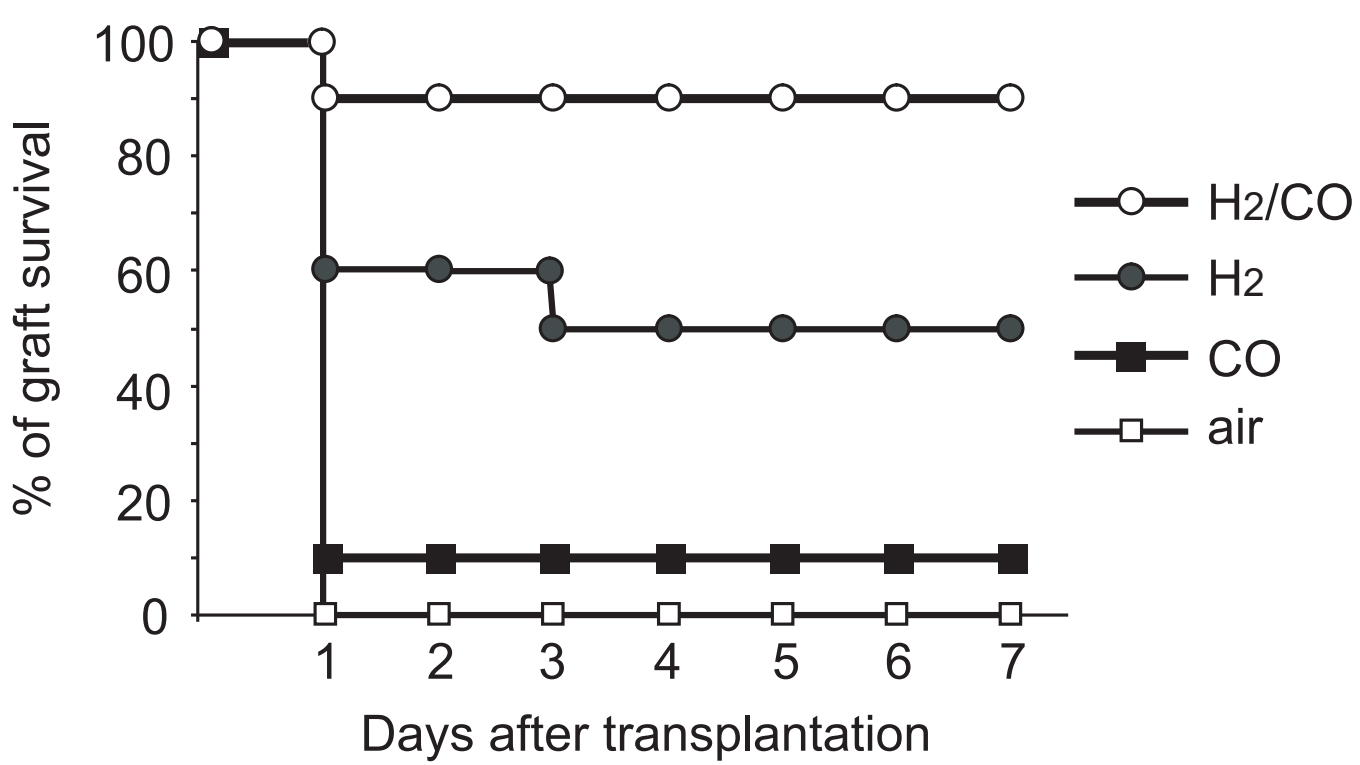
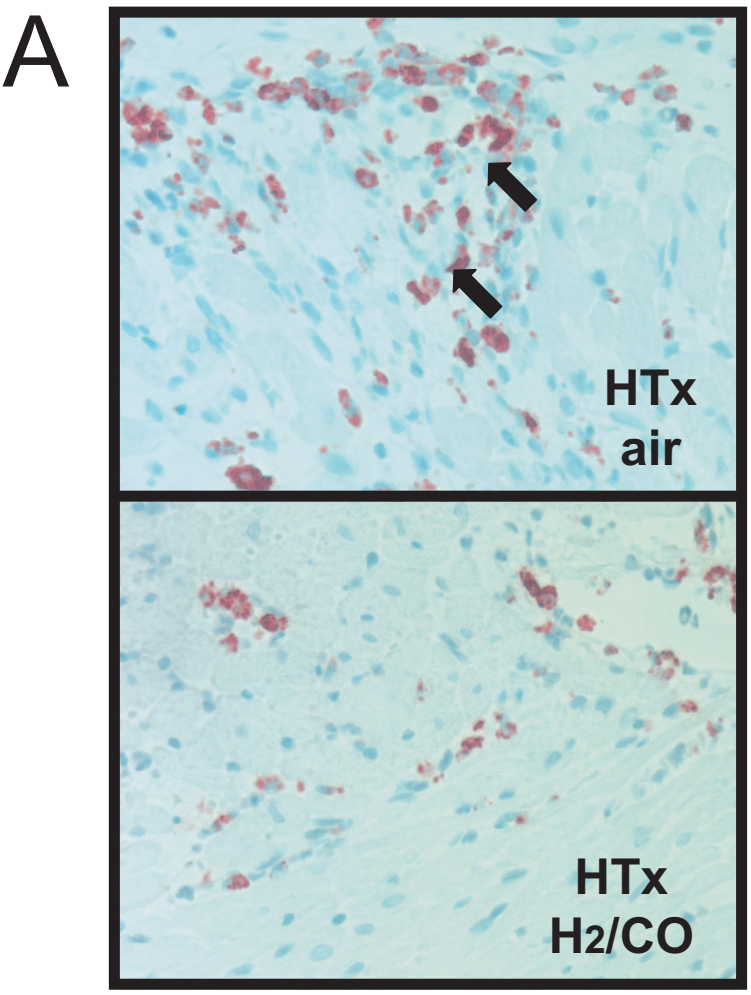
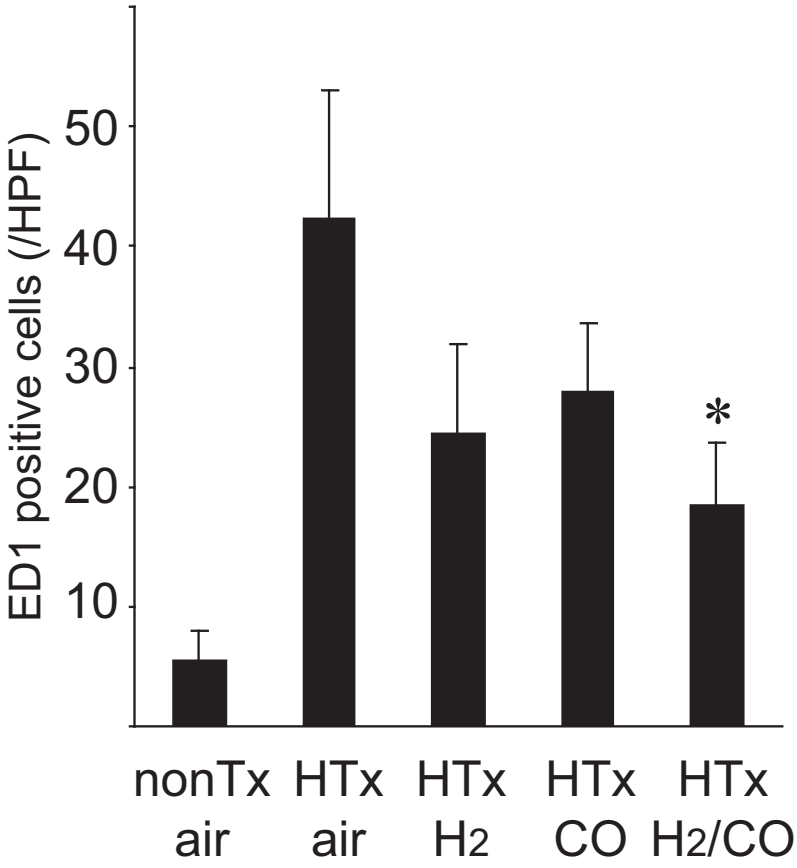
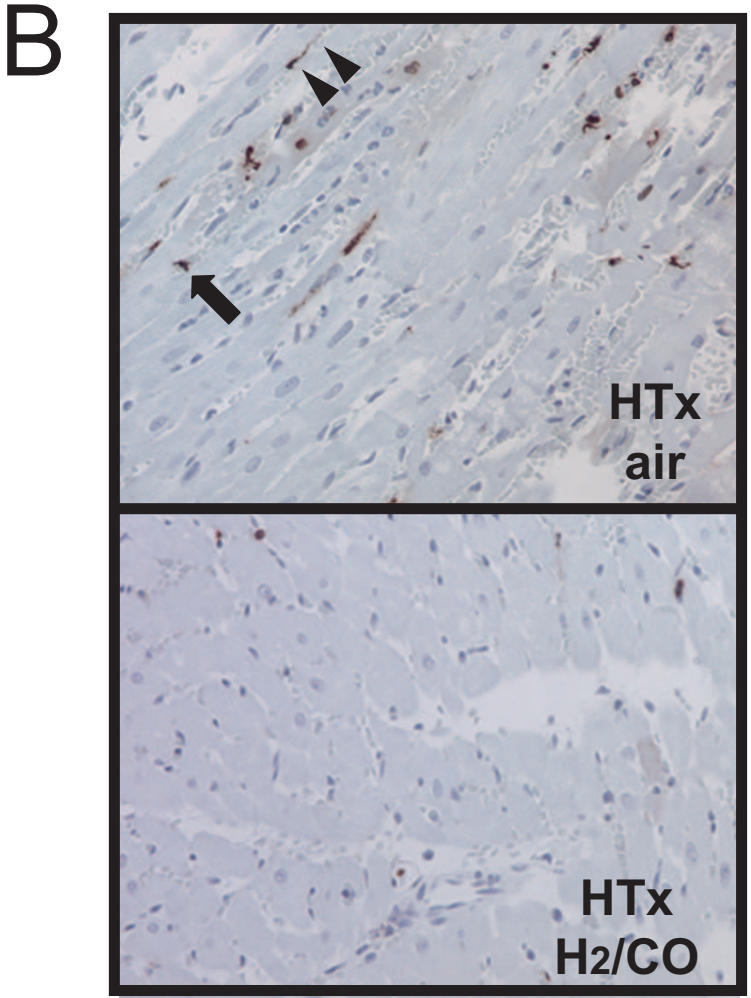

% of graft survival
0
20
40
60
80
100
1
2
3
4
5
6
7
Days after transplantation
H2/CO
H2
CO
air

Figure 3


A
HTx
air
HTx
H2/CO


ED1 positive cells (/HPF)
50
40
30
20
10
*
nonTx air
HTx air
HTx H2
HTx CO
HTx H2/CO


B
HTx
air
HTx
H2/CO

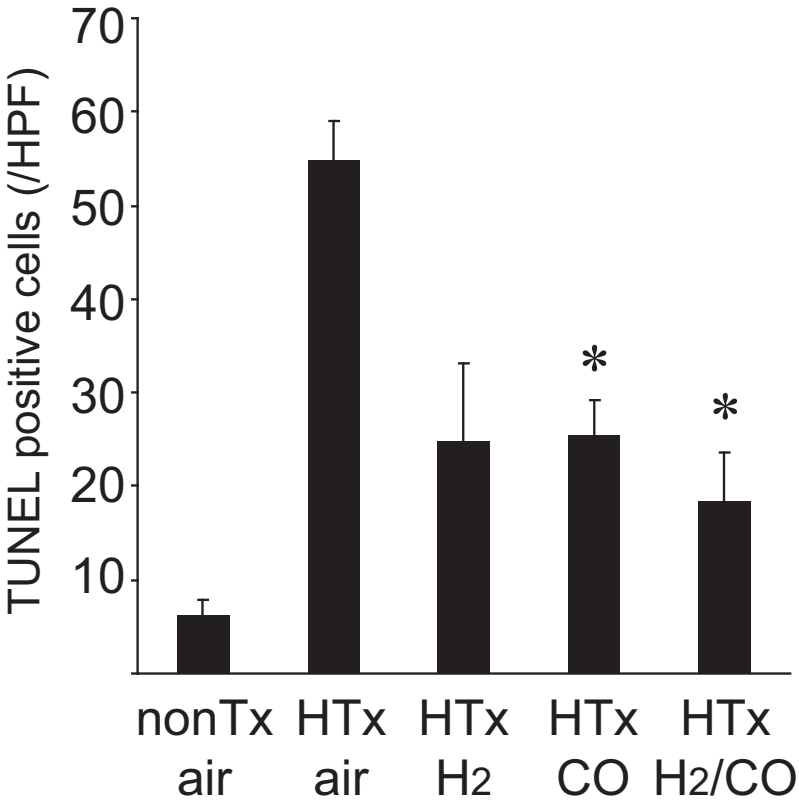

TUNEL positive cells (/HPF)
70
60
50
40
30
20
10
*
*
nonTx air
HTx air
HTx H2
HTx CO
HTx H2/CO

Figure 3

C

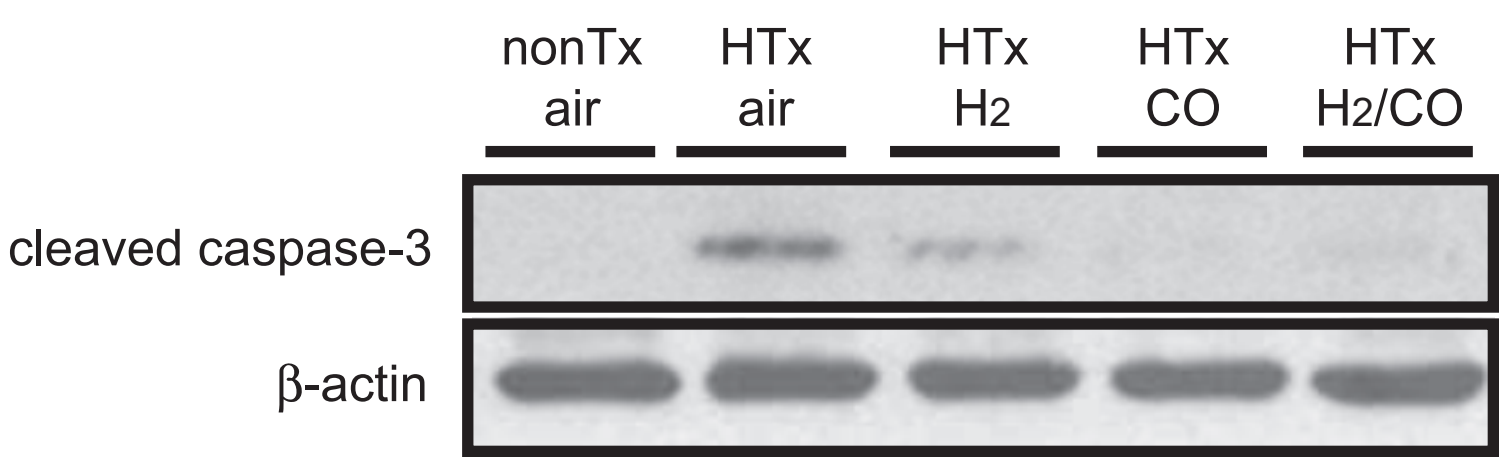

nonTx
air
HTx
air
HTx
H2
HTx
CO
HTx
H2/CO
cleaved caspase-3
β-actin

Figure 4

Figure 4

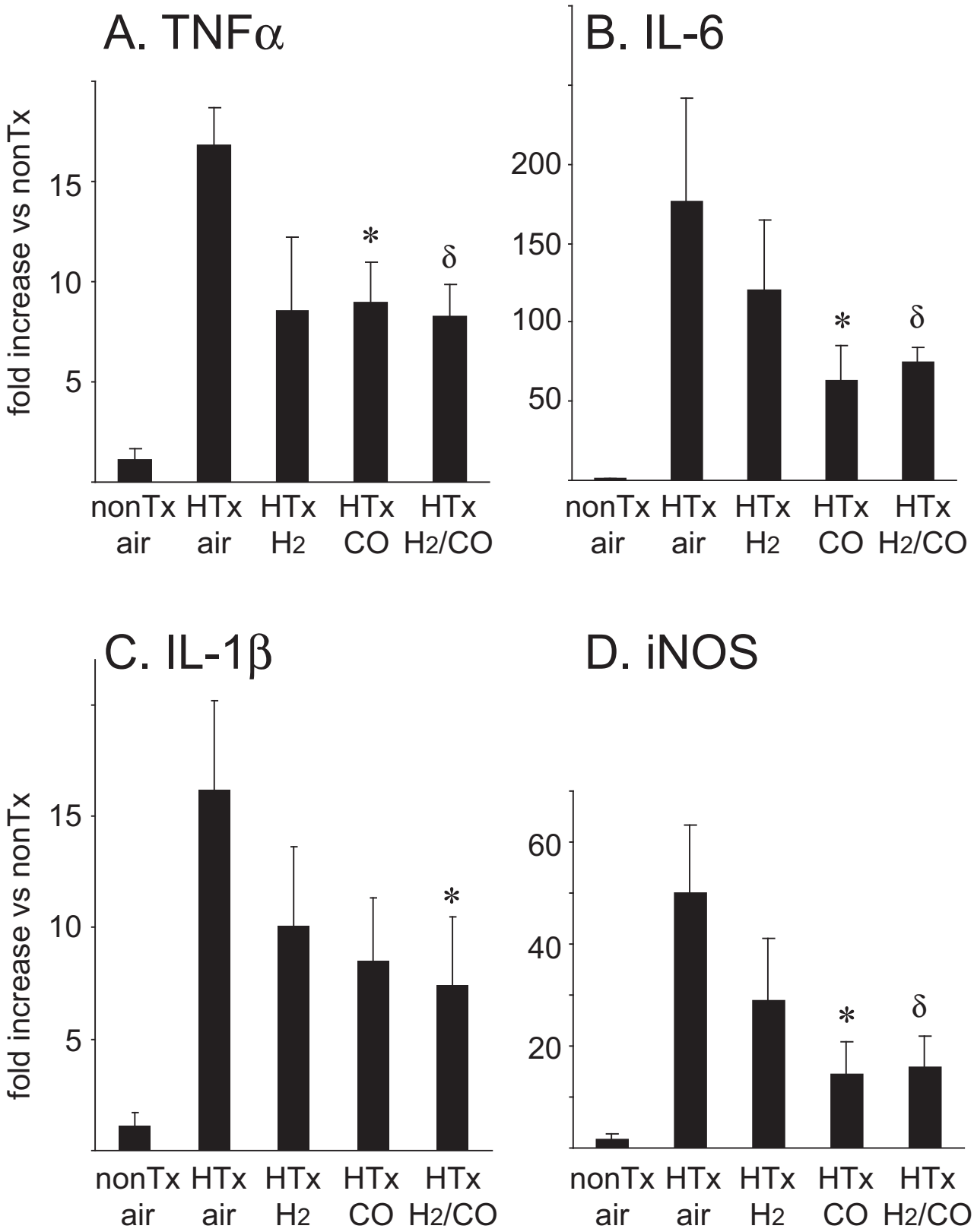


Figure 5

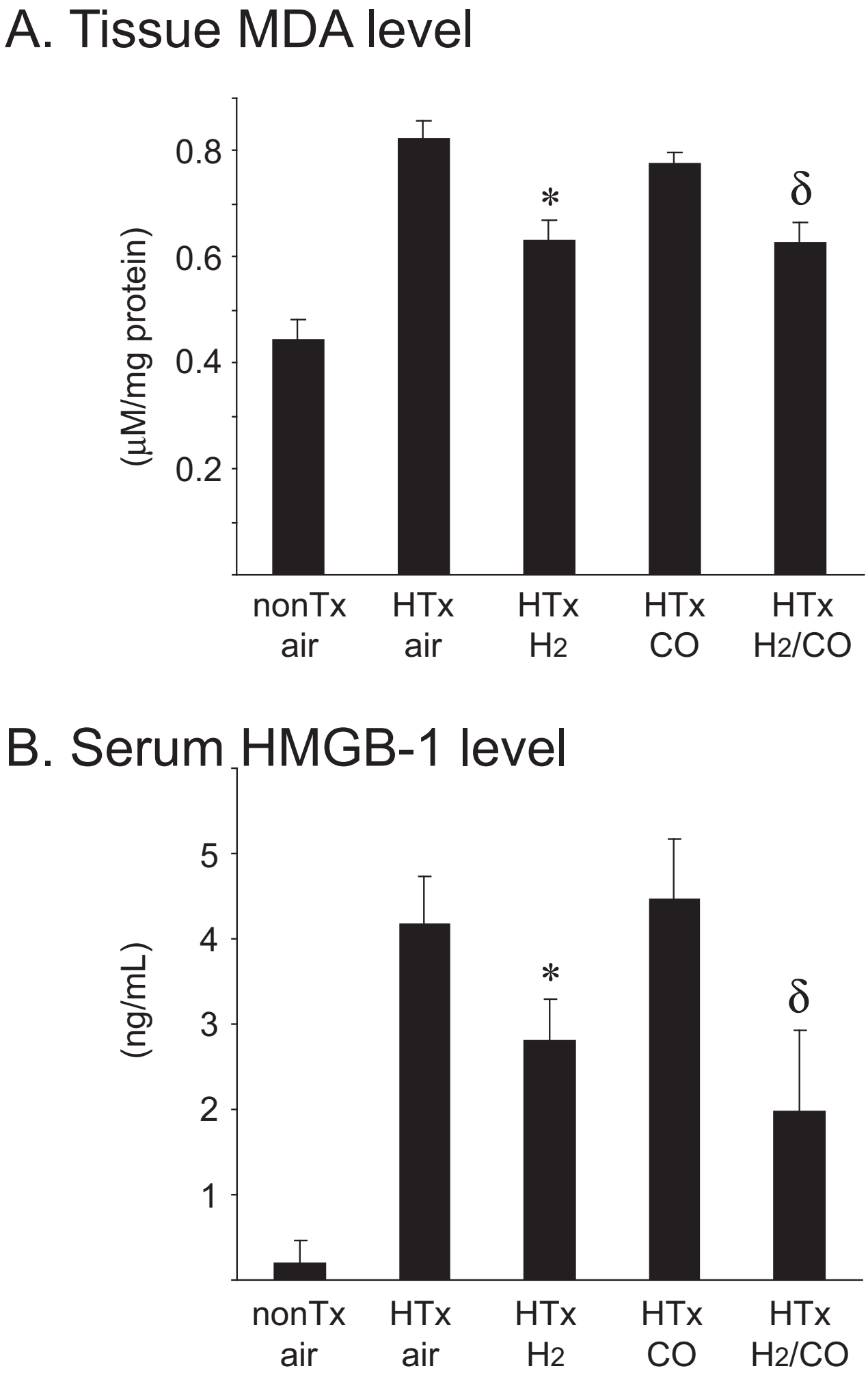
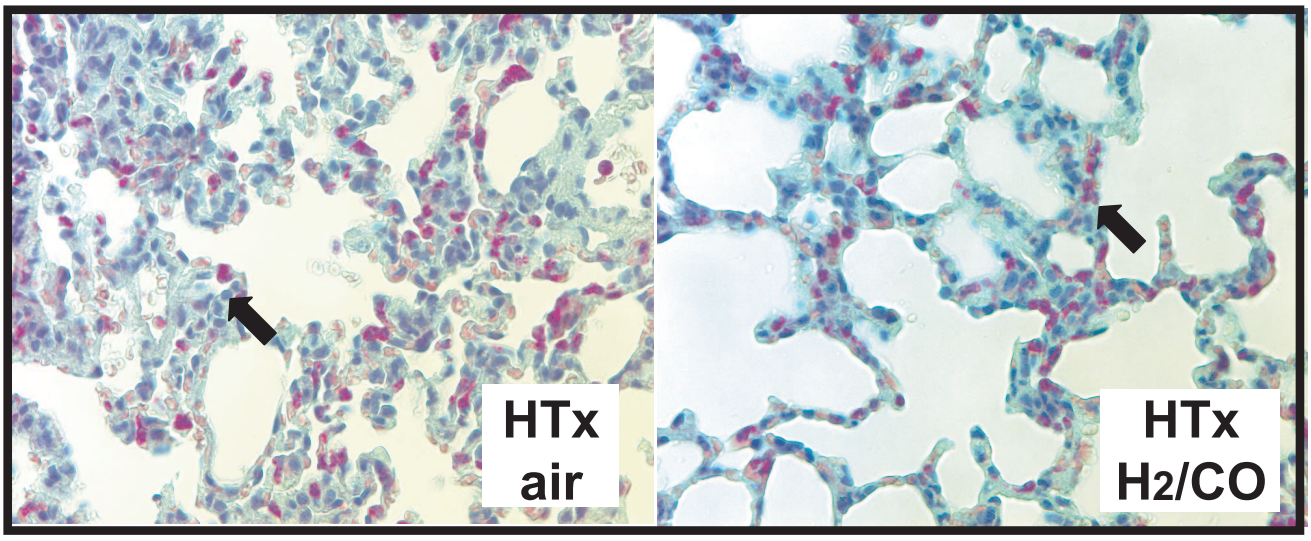
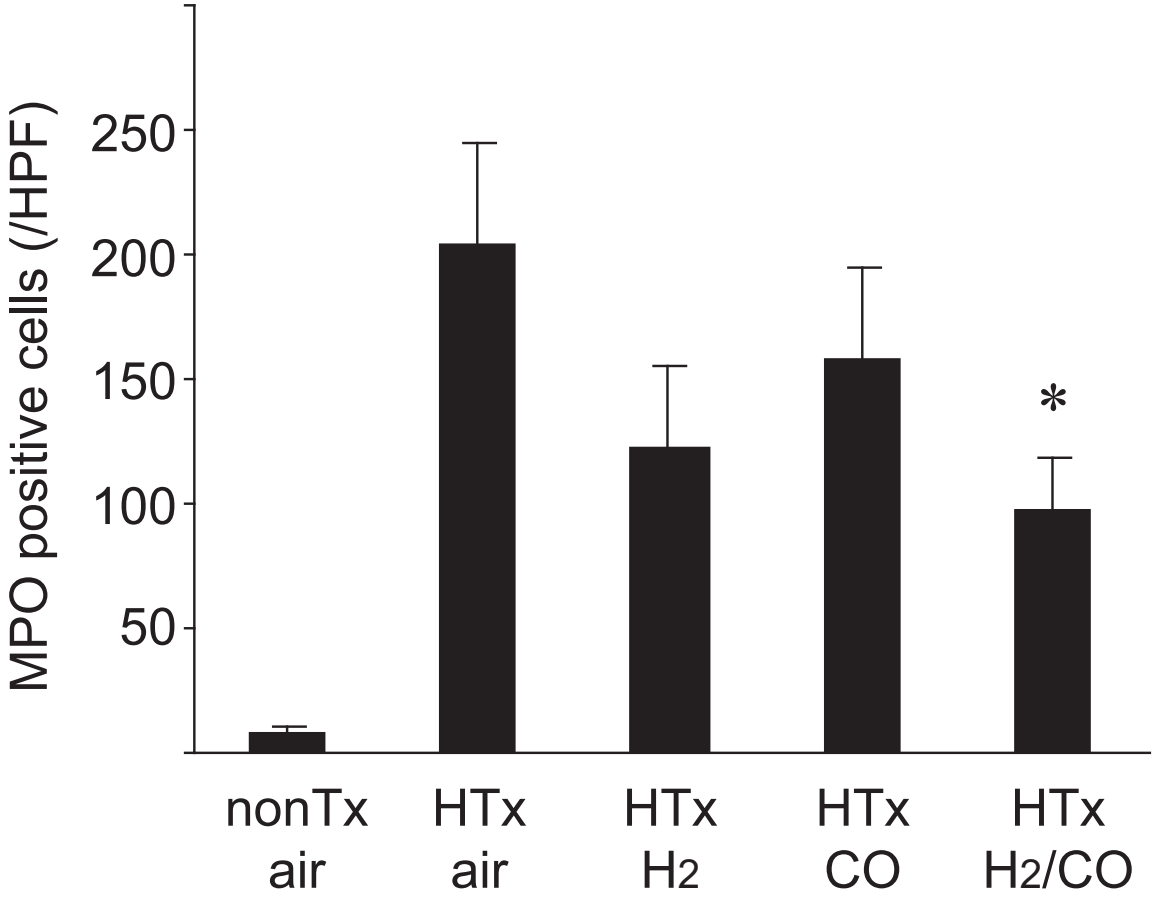

A. Tissue MDA level
(μM/mg protein)
0.8
0.6
0.4
0.2
*
δ
nonTx air
HTx air
HTx H2
HTx CO
HTx H2/CO
B. Serum HMGB-1 level
(ng/mL)
5
4
3
2
1
*
δ
nonTx air
HTx air
HTx H2
HTx CO
HTx H2/CO

Figure 6

A


HTx
air
HTx
H2/CO

B


MPO positive cells (/HPF)
250
200
150
100
50
*
nonTx
air
HTx
air
HTx
H2
HTx
CO
HTx
H2/CO