

Authorship:

Y.N. mainly performed the experiments and wrote the draft. K.H. obtained the grant, designed the study protocol, participated in performing the research, analyzed the data, and wrote the draft. T.T. performed and/or supervised histological assays and analyses. I.T., Y.O., H.M., J.K., T.T., H.H., T.K., O.I., J.Y., T.G., and H.T. participated in performing the research and assays. S.U. supervised the research, participated in data analyses, and revised the draft.

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The authors declare no conflicts of interest.

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Abbreviations:

ALT, alanine aminotransferase; ANOVA, analysis of variance; ANP, atrial natriuretic peptide; AST, aspartate aminotransferase; ATP, adenosine triphosphate; CS, cold storage; DBD, donor after brain death; DCD, donors after circulatory death; ECD, extended-criteria donor; ET-1, endothelin-1; G, gauge; HA, Hyaluronic acid; hANP, human atrial natriuretic peptide; HSC, hepatic stellate cell; HTK, histidine-tryptophan-ketoglutarate; IPRL, isolated perfused rat liver; IRI, ischemia/reperfusion injury; KHB, Krebs-Henselite buffer; LDH, lactate dehydrogenase; LT, liver transplantation; MP, machine perfusion; NO, nitric oxide; NOS, Nitric Oxide Synthase; PNF, primary non-function; PV, portal vein; PVP, portal venous pressure; qRT-PCR, quantitative Real-Time Polymerase Chain Reaction; ROS, reactive oxygen species; SEC, sinusoidal endothelial cells; TNF- α , tumor necrosis factor- α ; UW, University of Wisconsin solution; 8-OHdG, 8-hydroxy-2-deoxyguanosine

Abstract:

Background: Current critical shortage of donor organs has increased the use of donation after circulatory death (DCD) livers for transplantation, despite higher risk for primary non-function or ischemic cholangiopathy. Human atrial natriuretic peptide (hANP) is a cardiovascular hormone that possesses protective action to vascular endothelia. We aimed to clarify the therapeutic potential of hANP in cold storage of DCD livers.

Methods: Male Wistar rats were exposed to 30-minute warm ischemia *in situ*. Livers were then retrieved and cold-preserved for 6 hours with or without hANP supplementation. Functional and morphological integrity of the livers was evaluated by oxygenated *ex vivo* reperfusion at 37°C.

Results: hANP supplementation resulted in significant reduction of portal venous pressure (12.2 ± 0.5 vs. 22.5 ± 3.5 mmHg, $P < 0.001$). As underlying mechanisms, hANP supplementation significantly increased tissue adenosine concentration ($P = 0.008$), followed by significant up-regulation of endothelial NOS and endothelin-1 down-regulation than in the controls ($P = 0.01$ and $P = 0.004$, respectively). Consequently, hANP significantly decreased transaminase release ($P < 0.001$), and increased bile production (96.2 ± 18.2 vs. 36.2 ± 15.2 $\mu\text{L/g-liver/hour}$, $P < 0.001$). Morphologically, hepatocytes and sinusoidal endothelia were both better maintained by hANP ($P = 0.021$). Electron microscopy also revealed that sinusoidal ultra-structures and microvilli formation in bile canaliculi were both better-preserved by hANP supplementation. Silver staining also demonstrated that hANP significantly preserved reticulin fibers in Disse space ($P = 0.017$), representing significant protection of sinusoidal frameworks/architectures.

Conclusions: Supplementation of hANP during cold storage significantly attenuated cold-ischemia/warm-reperfusion injury of DCD livers.

Introduction

Chronic and growing shortage of donor organs is a worldwide critical issue in organ transplantation, which has increased the use of donation after circulatory death (DCD) livers.^{1,2} Although utilization of DCD livers would substantially expand potential donor pools, it is very difficult to precisely estimate liver damage induced by DCD and subsequent hepatic function.

Nowadays, only limited transplant centers in some industrialized countries perform DCD-liver transplant (LT), coupled with currently-developed *ex vivo* machine perfusion techniques.^{3,4} Despite such innovative advancement, much concern still exists regarding the outcome after DCD-LT, in which the graft survival is approximately 10-15% lower compared to the donation after brain death (DBD)-LT, because of higher rates of primary non-function (PNF) and long-lasting biliary complications.^{5,6}

Hepatic ischemia/reperfusion injury (IRI) is one of the main culprits for the poor outcome in DCD-LT. Attenuation or prevention of IRI remains an important clinical challenge to improve recipient outcome in DCD-LT. Warm ischemia *in situ* induces macro- and micro-thrombosis within hepatic vasculatures, leading to microcirculatory failure. In addition, subsequent cold storage (CS) significantly deteriorates vascular/sinusoidal endothelia, such as retraction, intercellular detachment, and apoptosis.^{7,8} Such ischemic damages during warm ischemic time (WIT) and subsequent CS are further exacerbated upon reperfusion by reactive oxygen species (ROS) burst, Kupffer cell (KC) activation, up-regulation of adhesion molecules, and subsequent leukocytes infiltration, finally leading to irreversible microcirculatory failure.

Human atrial natriuretic peptide (hANP) is a cardiovascular hormone, produced and secreted by heart atrium. hANP plays a major role in maintaining volume-, as well as pressure-homeostasis in cardiovascular system, such as diuresis, natriuresis, and

vasodilation.⁹ Several investigations have demonstrated its hepatocellular protection against hypoxia/reoxygenation.^{10,11} Furthermore, hANP preserves lung,¹² heart,¹³ and kidney¹⁴ functions after warm ischemia. Most recently, perioperative hANP administration in patients undergoing resection of lung cancer significantly lowered the recurrence rate of hematogenous metastasis by suppressing inflammatory reactions in vascular endothelia.¹⁵ In addition, interaction between hANP and hepatic endothelium was reported by a few investigations: hANP attenuates endothelin (ET)-1 up-regulation from hepatic stellate cells,¹⁶ and down-regulates Na⁺/H⁺ and Ca⁺/H⁺ ions exchangers in hepatocytes, thus reducing cell swelling during hypoxia.^{10,17} ET-1 plays a major role in regulating sinusoidal blood flow by hepatic stellate cells (HSCs) contraction.¹⁸

Based on these available evidence, we hypothesized that hANP supplementation into preservation solution might protect vascular endothelia, attenuate pro-inflammatory signals/cascades therein, and alleviate sinusoidal constriction, thereby improving damages and functions of DCD livers. This study was thus designed to evaluate the therapeutic potential of hANP on maintaining hepatic tissue integrity and function of cold-preserved DCD rat livers.

Materials and methods

Animals

Male Wistar rats (250-300g, Japan SLC, Inc., Shizuoka, Japan) were used as liver donors. The rats were kept under specific pathogen-free conditions in a temperature- and humidity-controlled environment with a 12-hour light-dark cycle, and had a free access to rat chow and water. All animal experiments were conducted under a protocol approved by the Animal Research Committee of Kyoto University, in accordance with the “Guide for the Care and Use of Laboratory Animals” (NIH Publication No 86-23, revised 1985).

Rat DCD model

The rats were generally anesthetized with isoflurane (Escain, Mylan, Osaka, Japan) via a small-animal anesthetizer (MK-A110; Muromachi Kikai, Tokyo, Japan). After midline laparotomy with bilateral subcostal extensions, the liver was carefully mobilized from all ligamentous attachments. After heparinization (300 IU/rat; Mochida Pharmaceutical, Tokyo, Japan), cardiac arrest was induced by phrenotomy and subsequent bilateral pneumothorax, followed by clamping the descending aorta. Donor animals remained untouched thereafter for 30 minutes. Warm ischemic time (WIT) *in situ* was counted from aorta clamping.¹⁹ Low body temperature was prevented by an isothermal heating pad and by covering the body, enabling to keep approximately $32.0 \pm 0.3^{\circ}\text{C}$ throughout WIT.

Liver procurement

The rats were randomly assigned into two groups ($n = 8$ each), Group-hANP or -Control. First, splanchnic organs were flushed *in situ* from aorta with 50mL of ice-cold University of Wisconsin (UW) solution (Viaspan, Astellas, Tokyo, Japan) with or without 2.5 $\mu\text{g/mL}$ hANP (hANP[®], Daiichi-Sankyo Co., Ltd. Tokyo, Japan) supplementation (Group-hANP or -Control, respectively). During the initial washout, the superior and inferior vena cava (SVC and IVC, respectively) were both incised to avoid hepatic outflow obstruction. The procedures hereafter were detailed elsewhere.^{20,21} Briefly, the common bile duct was cannulated with a 24-gauge (G) polyethylene tube (TERUMO, Tokyo, Japan). The hepatic artery was ligated and portal vein (PV) was cannulated with a 14-G catheter (ArgyleTM, COVIDIEN, Tokyo, Japan). The liver was perfused again with gravity from PV with 10 mL of cold UW with or without 2.5 $\mu\text{g/mL}$ hANP supplementation. SVC was cannulated with a 14-G short stent for isolated perfusion. Livers were then excised, weighed ($14.0 \pm 0.2\text{g}$), and cold-stored at 4°C for 6 hours in the respective UW solutions.

Ex vivo Oxygenated Warm Reperfusion

The isolated perfused rat liver (IPRL) system has been detailed elsewhere.²⁰⁻²² Briefly, the perfusion circuit was pre-rinsed, and perfused with hemoglobin-free Krebs-Henseleite buffer (KHB; K3753, Sigma-Aldrich Inc., St. Louis, USA), supplemented with 1mg/L of hyaluronic acid (HA; CAS.9067-32-7, Wako Pure Chemical Industries, Japan). To account for slow re-warming during vascular reconstruction, all livers were transferred and immersed into saline at room temperature for 15 minutes just before reperfusion. Thereafter, the livers were positioned in a Plexiglas chamber, and perfused via PV at a constant flow of 3 mL/g-liver/min at 37°C for 120 minutes using a roller pump. The perfusion fluid was 140 mL of modified KHB (pH 7.4), saturated with Carbogen (95%O₂ + 5%CO₂). The perfusate pO₂ was maintained above 500mmHg throughout reperfusion. In- and out-flow perfusate was sampled at 0, 10, 30, 60, and 120 minutes of reperfusion. Bile production was monitored, and portal-venous perfusion pressure (PVP) was continuously measured by a calibrated vertical column connected to the PV catheter.^{20,21}

Liver enzymes

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) release into hepatic effluent were both determined with a standard spectrophotometric method with an automated clinical analyzer (JCA-BM9030; JEOL, Ltd., Tokyo, Japan).

Oxidative stress

The 8-hydroxy-2-deoxyguanosine (8-OHdG) is a useful marker to estimate oxidative damage to liver tissues, mainly by hydroxyl radicals.²³ Liver effluent was analyzed with a high-sensitive 8-OHdG enzyme-linked immunosorbent assay (8-OHdG check, Nikken seil, Sizuoka, Japan) according to the manufacturer's protocol.

Hepatic oxygen consumption

Hepatic perfusate was sampled from both the portal inflow and the venous outflow, and oxygen contents were immediately determined using a blood gas analyzer (Rapid Point 405, Siemens, Tokyo, Japan). The oxygen consumption rate was calculated by the difference between the inflow and the outflow samples, and provided as $\mu\text{L/g-liver/minute}$ according to the transhepatic flow and liver mass.^{20,21}

Tissue adenosine triphosphate (ATP) & adenosine concentrations

At the end of IPRL, liver tissues were snap-frozen and stored in liquid nitrogen until later analysis. Tissue adenosine triphosphate (ATP) concentrations were determined by the luciferin-luciferase method with an ATP bioluminescence assay kit (Toyo B-Net Co., Ltd., Tokyo, Japan).²¹ Adenosine concentration in liver tissues was also measured with an enzyme-coupled fluorometric kit (Adenosine Assay Kit, Abcam, UK) according to the manufacturer's instruction.²⁴ The results were normalized to tissue protein concentrations, measured by BCA protein assay kit (Thermo Fisher Scientific K.K., Yokohama, Japan).

As a reference, normal, non-DCD livers, not subjected to warm ischemia but to 6-hour CS and subsequent 2-hour oxygenated reperfusion, were prepared in these assays to know the baseline values (Fig.2-B and 4-E, respectively), but not included in statistical analyses.

Hyaluronic acid (HA) clearance

To estimate the remaining viability of sinusoidal endothelial cells (SECs) of DCD livers, hyaluronic acid (HA) clearance was calculated.²⁵ Because SECs are the dominant site for uptaking and degrading HA,²⁶ and HA production from other organs is completely excluded in the current isolated setting, HA clearance in IPRL precisely estimates the functional integrity of SECs. The HA concentration in perfusate samples was measured with

enzyme-linked protein assay (Corgenix Inc., Colorado, USA) according to the manufacturer's protocol.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Total messenger-RNA (mRNA) was extracted from liver tissue after 120-min reperfusion using a NucleoSpin[®] RNA Kit (MACHEREY-NAGEL GmbH & Co. Kg, Düren, Germany). Equal amounts of mRNA were adjusted with NanoDrop2000 (NanoDrop Technologies, Washington, DE). Complementary DNA was reverse-transcribed using an Omniscript RT kit (Qiagen, Tokyo, Japan). Quantitative RT-PCR was performed with the following amplification conditions: 50.0°C for 2 seconds and 95.0°C for 10 seconds during holding stage followed by 45 cycles of 95.0°C for 0.15 seconds and 60.0°C for 1.0 second. PCR products were analyzed with the StepOnePlus[™] Real-Time PCR System (Applied Biosystems, Life Technologies Japan, Tokyo, Japan). Target gene expressions were calculated relative to the housekeeping gene, β -actin. TaqMan probes and primers for endotheli-1 (ET-1: assay ID Rn9999901_m1), tumor necrosis factor- α (TNF- α : ID Rn01410330_m1), endothelial Nitric Oxide Synthase (eNOS: ID Rn00562055_m1), inducible nitric oxide synthase (iNOS: ID Rn00561646_m1), and β -actin (ID Rn00667869_m1) were obtained from TaqMan gene expression assays (Applied Biosystems[™], Tokyo, Japan).

Bile production and LDH leakage into bile

Bile was collected throughout oxygenated perfusion, served as a functional parameter of DCD liver grafts. Lactate dehydrogenase (LDH) leakage into bile was also measured, given as a marker for biliary damages.^{21,27}

Light microscopy

At the end of reperfusion, tissue samples were obtained from the left lobe, stored and fixed in 4% paraformaldehyde, paraffinized, and sectioned to 4- μ m thickness. After hematoxylin-eosin staining, two independent pathologists assessed and graded hepatic tissue damage from 1 (excellent) to 4 (poor) in a blind fashion, according to the criteria reported elsewhere,²⁸ as follows: (1) normal rectangular structure, (2) rounded hepatocytes with increased sinusoidal spaces, (3) apoptosis (vacuolization and nuclear picnosis), and (4) necrosis. The scores were calculated from 10 independent observations (magnification x 400), and eight livers per group.

Electron microscopy

At the end of reperfusion, additional five livers from the both groups were perfused through PV with 2%-glutaraldehyde/4%-paraformaldehyde in 0.1M phosphate buffer, pH 7.4. After fixation, the samples were cut into 1-mm slices for scanning electron microscope (SEM) analysis. After postfixation with 1% osmium tetroxide, SEM samples were dehydrated, substituted, freeze-dried with t-butyl-alcohol, ion-sputter-coated, and then examined with S-4700 SEM (Hitachi High-Technologies, Tokyo, Japan).

Silver staining

Silver impregnation staining for reticulin fibers was used to assess sinusoidal wall architecture, such as collapse/disconnection of the reticulin framework in ischemically-damaged liver lobules.^{29,30} Ten randomly-selected fields from five livers per each group were examined under an optical microscope. The area of the reticulin network was quantified using Image J (version 1.6.0_22, NIH, USA).³¹ According to the instruction,³² the red channel was chosen on the software to emphasize reticulin fibers on the picture, thereby to binarize and quantify the reticulin fibers. The lower threshold was set as 55.

Statistical analysis

All statistical analyses were performed using Prism 6 (Graph Pad Software, Inc., San Diego, CA, USA). All values are expressed as the mean $\pm$ standard error of the mean (SEM). Two-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni's post-test was used to assess time-dependent parameters including PVP, transaminase release, and O₂ consumption. Statistical significance at each time-point was annotated as follows: *: $P < 0.05$; †: $P < 0.01$; ‡: $P < 0.001$.

The other comparisons between the two groups were performed by two-sided Student's *t* test or Mann-Whitney U test, as appropriate. $P < 0.05$ was considered significant.

Results

Transaminase release

As summarized in Figure 1, AST release was significantly lower in Group-hANP than that in Group-Control (159.3 ± 15.4 vs. 241.3 ± 21.4 U/L at 2 hours, respectively, $P < 0.001$, Fig.1-A). ALT (134.2 ± 14.2 vs. 223.3 ± 31.5 U/L, $P < 0.001$, Fig.1-B) also exhibited significant reduction throughout reperfusion, indicating substantially less hepatocellular damage, achieved by hANP supplementation.

Tissue oxygen consumption, ATP charge, and oxidative damages

Though oxygen consumption rate in Group-hANP was tended to be higher than in Group-Control, it could not reach statistical significance (Fig.2-A). In contrast, ATP restoration was significantly higher in Group-hANP than in the controls ($P = 0.0023$, Fig.2-B), indicating that hANP supplementation produced significantly better ATP charge with an equal amount of oxygen consumption, compared with untreated livers. The values in non-DCD livers was also presented in Fig.2-B just for a reference.

Oxidative tissue damages was quantified by measuring 8-OHdG release into the perfusate. As in Fig.2-C, hANP significantly lowered oxidative tissue damage than in Group-Control (0.54 ± 0.04 vs. 0.73 ± 0.04 ng/mL, $P < 0.05$).

Taken all these together, hANP supplementation enabled efficient oxygen utilization to restore ATP charge, while in controls, substantial amount of oxygen was consumed to generate oxidative stress.

Total vascular resistance and Sinusoidal endothelial function

During reperfusion, PVP, served as a parameter for total vascular resistance of DCD livers, showed time-dependent increase up to 22.5 ± 3.5 mmHg. In contrast, PVP in hANP-treated livers maintained significantly lower level, retaining 12.2 ± 0.5 mmHg at the end of reperfusion ($P < 0.001$, Fig.3-A).

Regarding the functional integrity of sinusoidal endothelia, HA clearance in Group-hANP was significantly higher than in Group-Control (2139.8 ± 257.9 vs. 1381.2 ± 237.1 ng/g-liver/hour, $P < 0.05$, Fig.3-B).

Morphologically, scanning electron microscopy revealed that sinusoidal endothelial linings in DCD livers were severely deteriorated, and sinusoidal pores were fused and enlarged (Fig.3-C), whereas hANP supplementation significantly attenuated such deleterious alterations in sinusoidal endothelia (Fig.3-D).

Vasodilatory effects by hANP

To examine the mechanisms underlying lowered PVP achieved by hANP, mRNA signals of eNOS, a well-known vaso-relaxant, as well as of ET-1, a vaso-constrictor in hepatic sinusoids, were both determined by quantitative RT-PCR. Of interest, hANP significantly up-regulated eNOS from 0.36 ± 0.14 in the controls up to 0.86 ± 0.10 ($P < 0.05$,

Fig.4-A), while ET-1 was significantly down-regulated to 35.1% of the controls (1.48 ± 0.25 vs. 0.52 ± 0.05 , $P < 0.01$, Fig.4-B).

Tissue adenosine concentration in Group-hANP was significantly higher than that in Group-Control ($P = 0.008$). Of interest, the value in Group-hANP showed higher tendency even compared with normal, non-DCD livers (Fig.4-E), indicating that hANP strongly up-regulates tissue adenosine concentration.

Anti-inflammatory effects by hANP

As shown in Figure 4-C and -D, mRNA expressions of TNF- α and iNOS were both significantly down-regulated in Group-hANP than in Group-Control (1.26 ± 0.06 vs. 1.79 ± 0.16 , $P < 0.05$, and 0.66 ± 0.13 vs. 1.41 ± 0.30 , $P < 0.05$, respectively), directly reflecting strong anti-inflammatory effect by hANP.

Bile production and Biliary Damage

Total remaining function of DCD livers was appraised by bile production during reperfusion. As displayed in Fig.5-A, bile volume in Group-hANP was 3-fold more than that in Group-Control (96.2 ± 18.2 vs. 36.2 ± 15.2 $\mu\text{L/g-liver/2 hours}$, $P < 0.001$).

Comprehensive biliary damage was estimated by LDH leakage into bile.^{21,27} In the controls, bile LDH was as very high as 1916.8 ± 331.9 IU/L, which was significantly improved by hANP to less than half (932.0 ± 194.3 , $P < 0.05$, Fig.5-B).

Microvilli formation in bile canaliculi was significantly decreased and their height was also lowered in Group-Control (Fig.5-C). In contrast, hANP significantly preserved the density and height of microvilli (Fig.5-D).

Histopathological evaluation

Representative tissue sections of DCD livers after 6-hour CS and subsequent 2-hour oxygenated reperfusion are presented in Figure 6. As seen, hematoxylin/eosin staining revealed ballooning degeneration around pericentral zones, as well as scattering acidophilic bodies in the controls, representing necrosis and apoptosis of hepatocytes. Disturbed architecture of hepatic cords, sinusoidal dilatation, and focal necrosis were also found across hepatic lobules in untreated livers (Fig.6-A, and -C), however, these damages were attenuated by hANP supplementation (Fig.6-B and -D). Thus, tissue damage scoring demonstrated significant improvement of hANP-treated livers compared to the controls (10.9 ± 0.8 vs. 13.7 ± 0.7 , $P < 0.05$, Fig.6-E).

Silver staining

As shown in Fig.7-A to -F, silver staining of DCD liver tissues demonstrated that reticulin fibers were better-maintained in Group-hANP compared with Group-Control. The relative ratio of reticulin fibers in Group-hANP was significantly higher than in Group-Control (4.8 ± 0.3 vs. 3.3 ± 0.4 , $P < 0.05$, Fig.7-G), highlighting the substantial disruption of fibrous ultrastructure constituting sinusoidal architectures in DCD livers, as well as significant amelioration thereof achieved by hANP supplementation.

Discussion

Cells, tissues, and organs communicate with each other to maintain or regulate surrounding circumstances for their better physiological functions. Various hormones, cytokines, and chemokines are involved in such endocrine homeostasis under not only physiological, but also diverse pathological conditions. One of such warning signals from the heart is ANP, which exerts several biological action to reduce cardiac over-load, including

vaso-dilatation, diuresis, and natriuresis.³³⁻³⁵ Besides its remarkable cardio-protective properties, much attention has recently been paid to its protective action to vascular endothelia.³⁶ Emerging evidence has shown that peri-operative hANP administration just for three days attenuates inflammatory reactions in endothelial cells, such as E-selectin up-regulation, inhibits adhesion of circulating tumor cells to vascular endothelia in remote organs, thereby preventing hematogenous metastasis for years.³⁷ In addition to its direct action to vascular endothelia, immunomodulatory effects of hANP was also reported, such as down-regulation of Kupffer cells activation in hepatic IRI.³⁸ Moreover, ANP was also proven to regulate p38-MAPK activation in a porcine warm IRI model.³⁹

These available evidence led us to hypothesize that hANP supplementation during preservation would possibly exert cyto-protective effects on DCD liver grafts, in which extremely-large vascular beds in the liver are all severely pre-damaged by warm, as well as cold IRI.

As expected, we demonstrated for the first time that hANP supplementation could effectively attenuate IRI in DCD rat livers. The protection by hANP was associated with significant down-regulation of ET-1 and iNOS, both of which led to increase in vascular resistance of the liver. Of endothelium-derived vasoconstricting factors, ET-1 plays a pivotal role in contracting sinusoidal microcirculation.⁴⁰ In the present study, ET-1 mRNA was up-regulated in DCD livers, which was significantly down-regulated by hANP supplementation to one third of the control value. Görbig *et al.*¹⁶ reported that ANP blocks the influx of intracellular calcium ion, thereby directly inhibiting ET-1-induced HSC contraction. In addition to such direct action to ET-1/HSC, hANP attenuated iNOS up-regulation that further elicits ET-1 over-expression from HSCs, KCs, and SECs.⁴¹ Moreover, hANP supplementation also resulted in 2.4-fold increase in eNOS expression. Under physiological conditions, vasoactive nitric oxide (NO) is produced through eNOS in SECs and then diffuses

into Disse space, promoting HSCs relaxation and subsequent sinusoidal dilatation. The bioavailability of eNOS remains crucial to maintain the balance between the vaso-dilatory and -constrictive forces.⁴² As well-demonstrated, tissue adenosine up-regulation confers cytoprotection to ischemic tissue by inhibiting ET-1, as well as by increasing NO production, thereby improving microcirculatory disturbance.⁴³⁻⁴⁵ In the present study, we demonstrated that hANP supplementation significantly increased tissue adenosine concentration in DCD livers, concomitant with significant down-regulation of ET-1 and up-regulation of eNOS. Surprisingly, the value in hANP-treated DCD livers showed higher tendency than in normal, non-DCD controls. Such adenosine enhancement by hANP contributed to reduce hepatic vascular resistance in DCD livers, as evidenced by lower PVP.

Furthermore, HA clearance and electron microscopy revealed that hANP significantly preserved functional and morphological viability of sinusoidal endothelia in DCD livers. Thus, hANP supplementation significantly lowered high vascular resistance of DCD livers down to the half in control livers. Moreover, silver-staining of reticulin fibers also revealed better maintenance of ultrastructural architecture in sinusoidal framework by hANP. All these beneficial effects by hANP contributed with each other to protect sinusoidal wall structures comprising several components of SECs, HSCs and their multiple processes, nervous fibers, and extracellular matrixes, thereby ameliorating hepatic microcirculation in DCD livers.

Of interest, oxygen consumption rate in hANP-treated livers was similar to that in controls. Tissue ATP restoration after oxygenated reperfusion, however, exhibited double-fold improvement compared to controls. Such disparity between tissue oxygen uptake and resultant ATP charge may be attributed to the differences in simultaneous oxidative stress produced therein. In Group-Control, 8-OHdG, one of the most sensitive indices for oxidative cellular damages, was 33% higher than in hANP-treated livers, indicating that substantial amount of delivered oxygen was consumed to generate reactive oxygen species, not to restore

tissue energy charge. In other words, hANP supplementation efficiently utilized delivered oxygen to restore ATP, thereby maintaining tissue integrity of DCD livers. We recently reported similar observations in macro-steatotic livers, in which subnormothermic machine perfusion preservation resulted in significantly higher ATP restoration with less oxygen uptake, whereas cold-stored fatty livers consumed more oxygen with significantly higher oxidative damage and less energy charge.²¹ These results may suggest a common pathology in pre-damaged ECD livers with less functional viability of hepatocellular mitochondria and sinusoidal microcirculation.

Of various disadvantages of DCD liver grafts, ischemic cholangiopathy is the leading cause of graft loss.^{46,47} Perioperative biliary hypoxia due to impaired microcirculation seems to be responsible for non-anastomotic strictures in intra-hepatic bile ducts for a prolonged period.⁴⁸ From this perspective, hANP supplementation significantly lowered LDH leakage into bile to less than half of that in untreated livers, directly reflecting significantly-less biliary damage. This was followed by significantly more bile production, increasing up to 4-fold higher bile secretion compared to the control. Early bile flow upon reperfusion is one of the most reliable parameter for graft injury, particularly for microcirculatory disturbance.⁴⁹ Moreover, electron microscopy revealed that both density and height of microvilli in bile canaliculi was significantly better maintained by hANP. Although longer observation using a large animal model is required, these results suggest a therapeutic potential of hANP supplementation to ameliorate ischemic cholangiopathy of DCD-LT.

Since the first clinical application in 1995, hundreds of thousands of patients with congestive heart failure have received hANP administration. Because hANP is an endogenous hormone secreted by heart atrium, its unobtainable safety is very attractive for “drug repositioning” of this cardio-protective agent to different medical fields. Because the half-life of ANP in physiological blood circulation is approximately 2 minutes in human (4

minutes in rats),⁵⁰ continuous intravenous administration is necessary to sustain its therapeutic effect in *in vivo* conditions. However, a previous study demonstrated its sustainable effect under low temperature, exerting renal protection during prolonged CS.⁵¹ Consistently, we also demonstrated significant protection of DCD liver grafts by hANP supplementation during CS. Such “*ex vivo*” treatments to isolated organs enable to avoid even relatively-rare systemic adverse effects of this agent, such as hypotension.

As a limitation, this study adopted the *ex-vivo* reperfusion model that has been widely-used in various research fields including transplantation.^{20,21,27,52} This isolated setting enables us to avoid interferences from immunological alloreaactions and technical dispersions in liver transplant surgery in rodent, thereby allowing to assess organ preservation quality more precisely. However, validation of this novel preservation technique in a liver transplant model is prerequisite before the next step towards its clinical translation.

In conclusion, hANP supplementation during preservation significantly protected DCD liver grafts from IRI by ameliorating pre-damaged hepatic microvasculature by warm and cold IRI. Given its simplicity and unobtainable safety, hANP supplementation may be a novel therapeutic option for DCD liver grafts, which is easily applicable worldwide, even though highly-equipped MP devices are not available.

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Figure Legends

Figure 1: Hepatocellular damages upon reperfusion

AST (A) and ALT (B) releases into perfusate, as the indices for hepatocellular damage upon reperfusion. The difference between the groups was assessed by 2-way repeated-measurement ANOVA (AST: $P < 0.001$ and ALT: $P < 0.001$), followed by time-point assessment with Bonferroni's post-tests. *: $P < 0.05$; †: $P < 0.01$; and ‡: $P < 0.001$ vs. Group-Control ($n = 8$ each).

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ANOVA, analysis of variance.

Figure 2: Oxygen consumption, ATP restoration, and oxidative tissue damage upon reperfusion

(A) During reperfusion, hepatic oxygen consumption rate was calculated from the inflow- and the outflow- pO_2 , transhepatic flow, and the liver mass (mean $\pm$ SEM, $n = 8$ each). The inter-group difference by 2-way repeated-measurement ANOVA: $P = 0.08$.

(B) Hepatic tissue concentrations of adenosine triphosphate (ATP) as a parameter for functional recovery of hepatic mitochondria and resultant tissue integrity after 6 hours of cold storage followed by two hours of oxygenated reperfusion. Data are expressed as nanomoles per gram-protein. *: $P = 0.0023$ vs. Group-Control by unpaired Student's t test (mean $\pm$ SEM, $n = 8$ each). The baseline ATP in normal, non-DCD controls, not subjected to warm ischemia but to 6-hour CS and subsequent 2-hour oxygenated reperfusion, was also presented just for a reference.

(C) Determination of 8-OHdG release into perfusate, served as a marker for tissue oxidative damage. *: $P < 0.05$ by unpaired Student's t test (mean $\pm$ SEM, $n = 8$ each).

Abbreviations: ATP, adenosine triphosphate; ANOVA, analysis of variance; SEM, standard error of the mean; 8-OHdG, 8-hydroxy-2-deoxyguanosine.

Figure 3. Total vascular resistance, sinusoidal function, and endothelial damages upon reperfusion

(A) Time-course of PVP during 2-hour IPRL, as an index for total vascular resistance upon reperfusion. PVP was continuously monitored by a calibrated vertical column connected to the PV catheter throughout reperfusion. All data are given as the mean $\pm$ SEM in 8 livers per each group. The difference between the groups was assessed by 2-way repeated-measurement ANOVA ($P < 0.001$), followed by Bonferroni's post-test: †: $P < 0.01$ and ‡: $P < 0.001$ vs. Group-Control.

(B) Clearance rate of HA, as one of the most sensitive parameter for preserved function of SECs. *: $P < 0.05$ by unpaired Student's t test (mean $\pm$ SEM, $n = 8$ each).

(C and D) Representative photomicrographs of hepatic sinusoids observed by scanning electron microscopy (original magnification: $\times 5,000$). In the control livers, SEC was severely disrupted, where sinusoidal fenestrae (white arrows) were fused and enlarged after 6-hour cold storage followed by 2-hour oxygenated reperfusion (C). In contrast, sinusoidal linings and pores therein were well-preserved in hANP-treated livers (D), demonstrating significant protection of sinusoidal endothelia by hANP supplementation. A scale bar in each panel indicates 1 μ m. Damages of sinusoidal endothelia were assessed from 10 randomly-selected observations, and 5 livers per group.

Abbreviations: PVP, portal venous pressure; IPRL, isolated perfused rat liver; ANOVA, analysis of variance; SEM, standard error of the mean; HA, hyaluronic acid; SECs, sinusoidal endothelial cells; DCD, Donation after Circulatory Death.

Figure 4. Quantitative RT-PCR and tissue adenosine concentration

(A and B) The gene expressions of sinusoidal vaso-dilator, eNOS (A) and vaso-constrictor, ET-1 (B) in liver tissues after 6-hour cold storage and subsequent 2-hour oxygenated reperfusion. eNOS was significantly up-regulated, whereas ET-1 was significantly down-regulated by hANP supplementation (*: $P < 0.05$ and †: $P < 0.01$ by unpaired Student's *t* test, Mean $\pm$ SEM, $n = 5$ each).

(C and D) The gene expressions of TNF- α and iNOS in liver tissues after 6-hour cold storage and subsequent 2-hour oxygenated reperfusion. Both pro-inflammatory cytokines were significantly lower in Group-hANP than in Group-Control (*: $P < 0.05$; †: $P < 0.01$, by unpaired Student's *t* test, Mean $\pm$ SEM, $n = 5$ each).

(E) Tissue adenosine concentrations were determined after 6-hour CS and 2-hour oxygenated reperfusion with enzyme-coupled fluorometric assay (mean $\pm$ SEM, $n = 5$ each). Data are expressed as μ mol/g-protein. Note that hANP supplementation significantly increased tissue adenosine concentration of DCD liver grafts than that in non-treated controls ($P = 0.008$ by Mann-Whitney U test). As a reference, the value in normal livers, not subjected to warm ischemia (non-DCD) but to 6-hour CS and subsequent 2-hour oxygenated reperfusion, was also presented.

Abbreviations: eNOS, endothelial Nitric Oxide Synthase; ET-1, endothelin-1; iNOS, inducible Nitric Oxide Synthase; TNF- α , tumor necrosis factor- α ; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of the mean.

Figure 5. Bile production, biliary damage, and morphological integrity of microvilli formation in bile canaliculi

(A) Cumulative bile production during 2-hour oxygenated reperfusion, as one of the most credible parameters for liver graft function. Data are expressed as microliters per gram of

liver per hour. The difference between the groups by 2-way repeated-measurement ANOVA: $P < 0.001$. Time-point assessment by Bonferroni's post-test: $\ddagger P < 0.001$ vs. Control (mean $\pm$ SEM, $n = 8$ each).

(B) LDH leakage into bile as a marker for cholangiocellular damage (mean $\pm$ SEM, $n = 8$ each). *: $P < 0.05$ vs. Control by unpaired Student's t test.

(C and D) Photomicrographs of representative ultrastructural observation of bile canaliculi in Group-Control (C) and in Group-hANP (D) with scanning electron microscopy ($n = 5$ each). As shown, microvilli formation in DCD livers were sparse, and their height was also lowered, manifesting significant ischemic damage of intrahepatic bile ducts (C). In contrast, both density and height of microvilli were significantly-better maintained by hANP supplementation (D). The original smagnification was $\times 8,000$. A scale bar in each panel represents $2\mu\text{m}$.

Abbreviations: ANOVA, analysis of variance; SEM, standard error of the mean; DCD, Donation after Circulatory Death.

Figure 6. Histological analysis and scoring

Representative tissue sections stained with hematoxylin-eosin after 6-hour cold storage (CS) and subsequent 2-hour oxygenated reperfusion.

(A and B) At lower magnification ($\times 200$), DCD livers preserved by CS exhibited deleterious vacuolization particularly around pericentral areas (A), whereas in hANP-treated livers, such tissue damages were significantly attenuated (B). A scale bar in each panel represents $100\mu\text{m}$.

(C and D) At high magnification ($\times 400$), hepatocellular vacuolization, nuclear shrinkage, and focal hepatocytes necrosis was more evident in CS-preserved livers (C), whereas such

detrimental alterations were significantly alleviated in hANP-preserved livers (D). A scale bar in each panel represents 50 μ m.

(E) Quantification of tissue damage according to Suzuki's histological criteria. Histological score was significantly lower in Group-hANP than in Group-Control (*: $P < 0.05$ by unpaired Student's t test, mean $\pm$ SEM). The scores were calculated from 10 independent observations (magnification $\times 400$), and eight livers per group.

Abbreviations: CS, cold storage; CV, centrilobular venule; DCD, Donation after Circulatory Death; PV, portal venule.

Figure 7. Silver staining of reticulin fibers in Disse space

Silver impregnation staining was used to assess the integrity of sinusoidal wall architecture, such as collapse and/or disconnection of reticulin frameworks in liver lobules.

(A and B) The original pictures of silver-stained liver sections in Group-Control (A) and in Group-hANP (B).

(C, D, E, and F) Area of reticulin networks was quantified using Image J software, by which the red channel was chosen on the software to emphasize, binarize, and thereby to quantify the reticulin fibers. The original magnifications were $\times 400$. The blue arrows point to reticulin fibers in sinusoidal area.

(G) Relative ratio of the silver-stained area in hepatic sinusoids was significantly better-maintained in Group-hANP than in Group-Control (*: $P < 0.05$ by Student's t test, mean $\pm$ SEM, $n = 5$ each), highlighting the substantial disruption of fibrous ultrastructures constituting sinusoidal architecture in DCD livers, and significant amelioration thereof achieved by hANP supplementation.

Abbreviations: DCD, Donation after Circulatory Death; CV, centrilobular venule

Figure 1

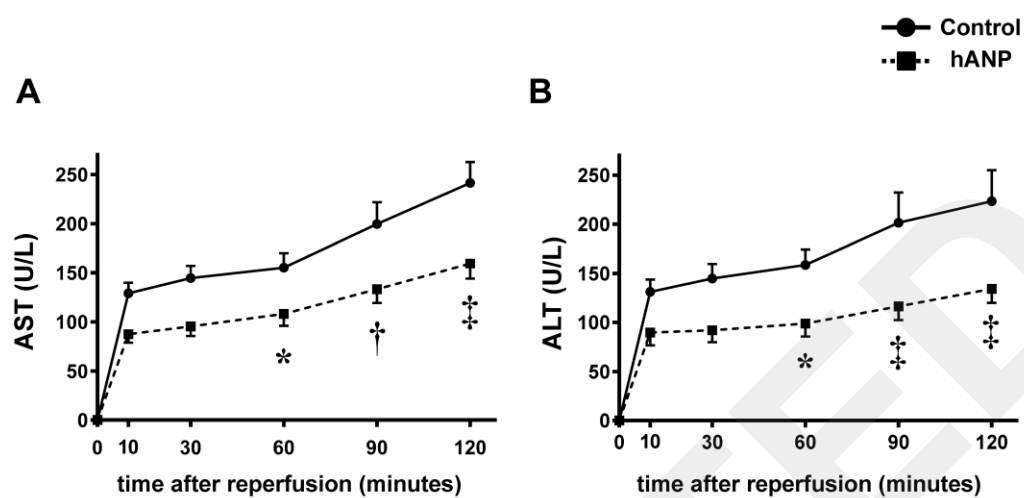


Figure 2

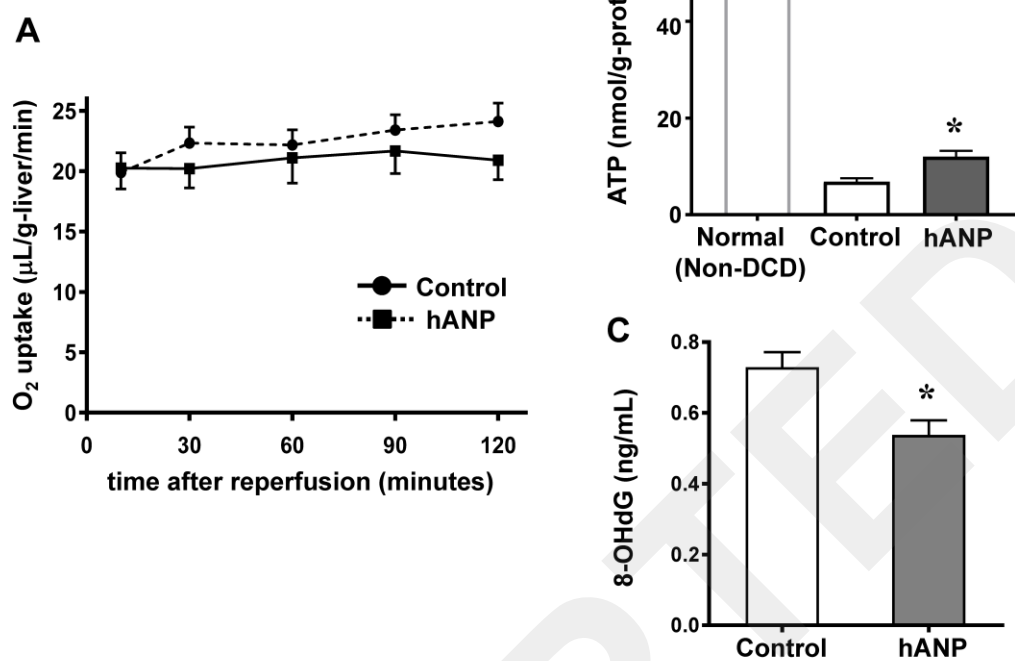


Figure 3

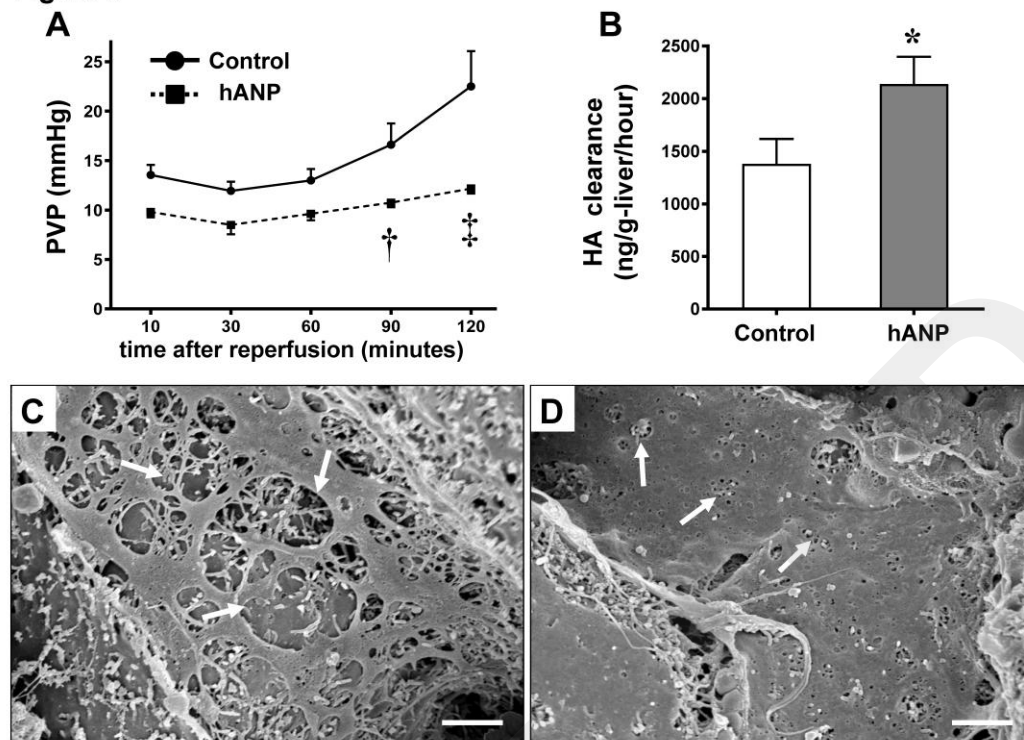


Figure 4

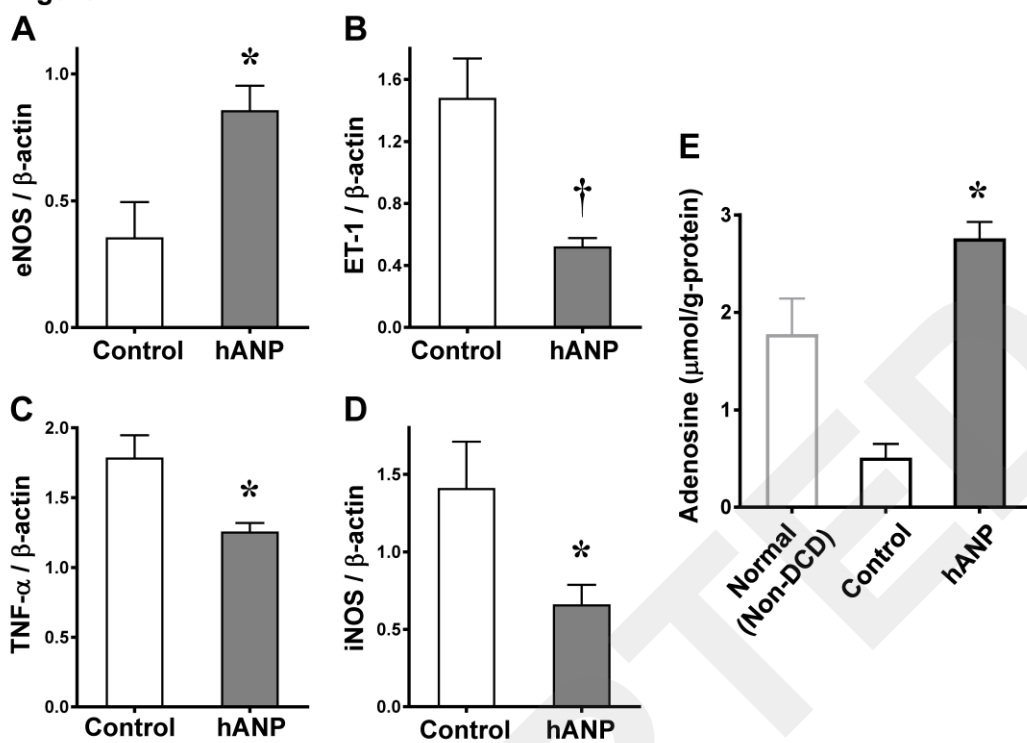


Figure 5

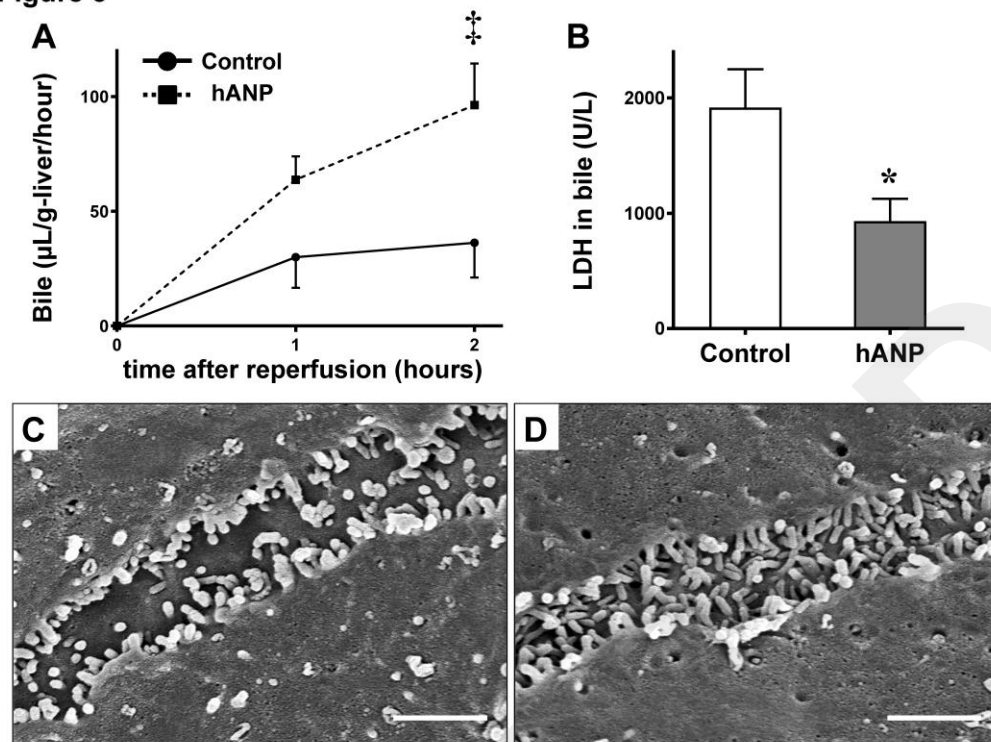


Figure 6

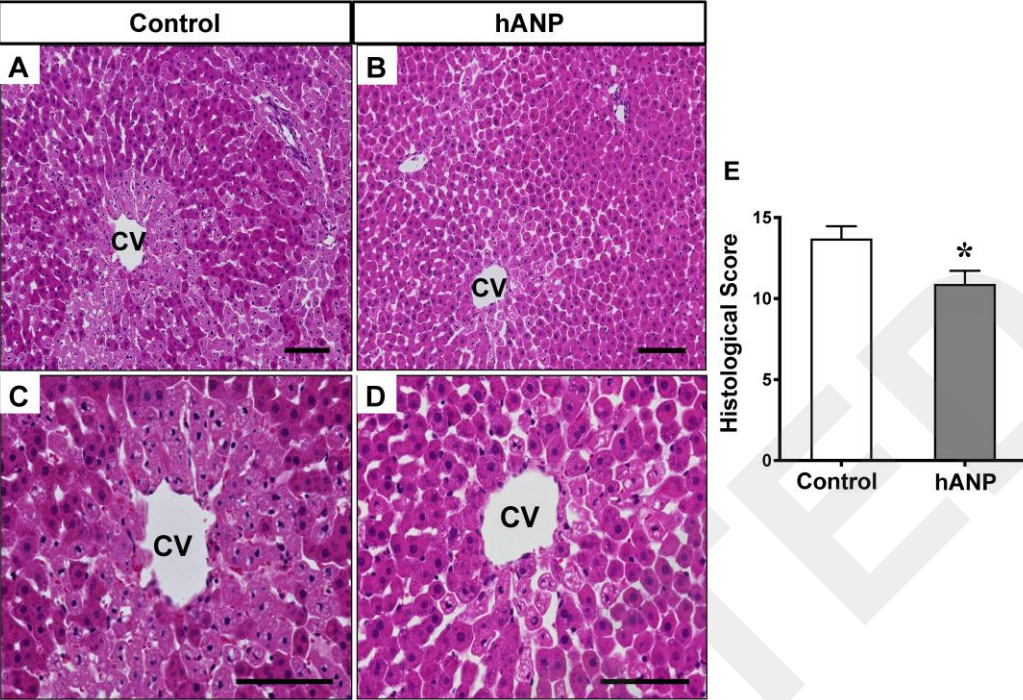


Figure 7

