SUPPLEMENTAL DATA

HYALURONAN MIXED ESTERS OF BUTYRIC AND RETINOIC ACID AFFORDING MYOCARDIAL SURVIVAL AND REPAIR WITHOUT STEM CELL TRANSPLANTATION*

Vincenzo Lionetti,[1,3,8] Silvia Cantoni,[2,8] Claudia Cavallini,[2] Francesca Bianchi,[2] Sabrina Valente,[7] Irene Frascari,[2] Elena Olivi,[2] Giovanni D. Aquaro,[3] Francesca Bonavita,[2] Ignazio Scarlata,[2] Margherita Maioli,[4] Valentina Vaccari,[2] Riccardo Tassinari,[2] Antonietta Bartoli,[5] Fabio A. Recchia,[1,6] Gianandrea Pasquinelli,[7] and Carlo Ventura[2]

From Sector of Medicine, Scuola Superiore S. Anna, Pisa, Italy[1], Laboratory of Molecular Biology and Stem Cell Engineering, Cardiovascular Department-National Institute of Biostructures and Biosystems, S. Orsola - Malpighi Hospital, University of Bologna, Bologna, Italy, and Bioscience Institute, Republic of San Marino[2], Institute of Clinical Physiology, CNR Fondazione G. Monasterio, Pisa, Italy[3], Department of Biomedical Sciences, University of Sassari, Sassari, Italy[4], Department of Physics, University of Pisa, Pisa, Italy[5], Department of Physiology, New York Medical College, Valhalla, NY, USA[6], and Department of Hematology, Oncology and Clinical Pathology, University of Bologna, Bologna, Italy[7]

Vincenzo Lionetti and Silvia Cantoni contributed equally to this work[8]

Running head: Endogenous Cell Therapy with a Synthetic Molecule

Address correspondence to: Carlo Ventura, MD, PhD, Laboratory of Molecular Biology and Stem Cell Engineering, Cardiovascular Department-National Institute of Biostructures and Biosystems, S. Orsola - Malpighi Hospital, University of Bologna, Via Massarenti 9, I-40138 Bologna, Italy. Fax/Tel: +39-051340339. E-mail: carlo.ventura@unibo.it or cvent@libero.it

EXPERIMENTAL PROCEDURES

Assessment of Apoptotic Cells- Apoptotic programmed cell death was assessed by TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End-Labeling) assay using the "*in situ* cell death detection kit, POD" (Roche Applied Science). Apoptosis evaluation was performed on histological sections sampled for immunohistochemical analysis. The TUNEL working procedure was carried out following the manufacturer's instruction. This kit reveals the "in situ" DNA strand breaks by labeling free 3'-OH termini with modified nucleotides through an enzymatic reaction. Briefly, the sections were dewaxed, rehydrated through a graded series of ethanol and rinsed in distilled water. Endogenous peroxidase was blocked by incubation in 3% H_2O_2 in methanol for 10 minutes at RT in the dark following washings in distilled water. All procedures were performed in a wet chamber. Antigenicity was recovered by enzymatic digestion with Proteinase K (DakoCytomation) for 30 minutes at 37°C. After washing, each sample was exposed to 50 µl of TUNEL reaction mixture containing enzyme solution, diluted 1:5 in TUNEL Dilution Buffer and Label Solution for 1 hour a 37°C in the dark. Then, the sections were incubated with the Converted POD followed by DAB (Sigma-Aldrich). Finally the specimens were counterstained with hematoxylin, dehydrated, and mounted. Positive controls were prepared using DNase to induce strand breaks for 10 minutes at RT before exposure to TUNEL reaction mixture. Negative controls were obtained by omitting enzyme solution from the labeling procedure.

For quantitative immunohistochemical analysis, digitalized images were acquired at 25x magnification (final magnification 250x), using the Image-Pro Plus® 6 software; cells expressing vWF, Stro-1 and c-kit molecules, stained with anti-acetyl-histone H4 and Ki-67 antibodies, or subjected to the TUNEL assay, were counted on a minimum of 10 randomly selected fields/each section; a minimum of 5 histological sections were examined for every sample and parameters were established to ensure that positive cells were counted only once. vWF positive capillaries and Stro-1, c-kit, H4, TUNEL positive cells were manually counted, while the areas of immunoreactions for H4, Ki-67, and VEGF were estimated by computer-assisted image analysis and their values expressed as the ratio of H4- or Ki-67 or VEGF-stained area to total area, as previously described (1).

Primer Sequence- Primers (0.25 μM) used were as follows: rat GAPDH forward 5'-ATGACAACTTTGGCATCGTG-3' and reverse 5'-GGATGCAGGGATGATGTTCT-3'; rat VEGF forward 5'-GGAGTACCCCGATGAGATAG-3' and reverse 5'-TATGTGCTGGCTTTGGTGAG-3'; rat Pim-1 forward 5'-CACGACGAAGAGATCGTCAA-3' and reverse 5'-CACGGATGGTTCTGGATTTC-3'; rat Akt forward 5'-ACTCATTCCAGACCCACGAC-3' and reverse 5'-CCGGTACACCACGTTCTTCT-3'; rat KDR forward 5'-TCCAGATGACAGCCAGACAG-3' and reverse 5'-GCTTTTACTGGGCATCATCC-3'; rat HGF forward 5'-ATGGCATTCCAACACAAACA-3' and reverse 5'-GTTTCTCCTCGCCTCTCTCA-3'.

REFERENCES

1. Lee, T.M., Lin, M.S., and Chang, N.C. (2007) *Am J Physiol Heart Circ Physiol* **293**(2), H968-H977

LEGEND OF VIDEO FILES

Representative left ventricular 1.5 T MRI images:

Supplemental Movie 1. Representative long axis images of untreated infarcted LV: myocardial contractility is globally depressed, with a more marked dysfunction in the anterior wall.

Supplemental Movie 2. Representative long axis images of infarcted LV treated with 100 ml of HBR solution (0.2 mg of HBR / 100 g of rat weight): myocardial contractility is globally preserved.

FIGURE LEGENDS

Fig. 1. HBR increased the number of capillary vessels, Stro-1-positive cells and perivascular elements. Full size images of each individual panel are presented. (A-C) Four weeks following myocardial infarction. Transversally cut left ventricular myocardium from HBR-treated hearts (100 μl of HBR solution, 0.2 mg of HBR / 100 g of rat weight) showed a reduced scar, compared with PBS-treated animals. (A, upper images, the arrows demarcate the infarcted area). Picro-Mallory stains in blue the area of scar and in red the myocardium parenchyma. In the border zone of HBR-treated hearts, scar reduction was associated with fewer apoptotic cardiomyocytes (A, lower images; scale bars = 20 μm), and increased number of capillary vessels (B). vWF expression highlights endothelial cells (arrows) lining the capillary inner wall (B); scale bars: upper images = 300 μm, lower images = 50 μm. (C) In HBR-treated samples, Stro-1 positive cells (arrows) increased in number and were closely associated with the outer capillary wall, while c-kit-positive cells did not vary significantly, compared to untreated animals. Scale bars = 20 μm. (D-F) 24 hours following myocardial infarction. (D) The number of Ki-67-positive cells significantly increased in the HBR-treated animals (arrows); scale bars = 50 μm. (E) Cells expressing NG2, and PDGF-Rβ (arrows); scale bars = 30 μm (upper images), and 100 μm (lower images). (F) VEGF expression (arrows). Scale bars = 100 μm (left and mid images), and 50 μm (right image). * Significantly different from PBS-treated hearts, $P < 0.05$. (Statistical test: two-tailed, unpaired Student's t-test). IHC: immunohistochemistry.

Figure 1A

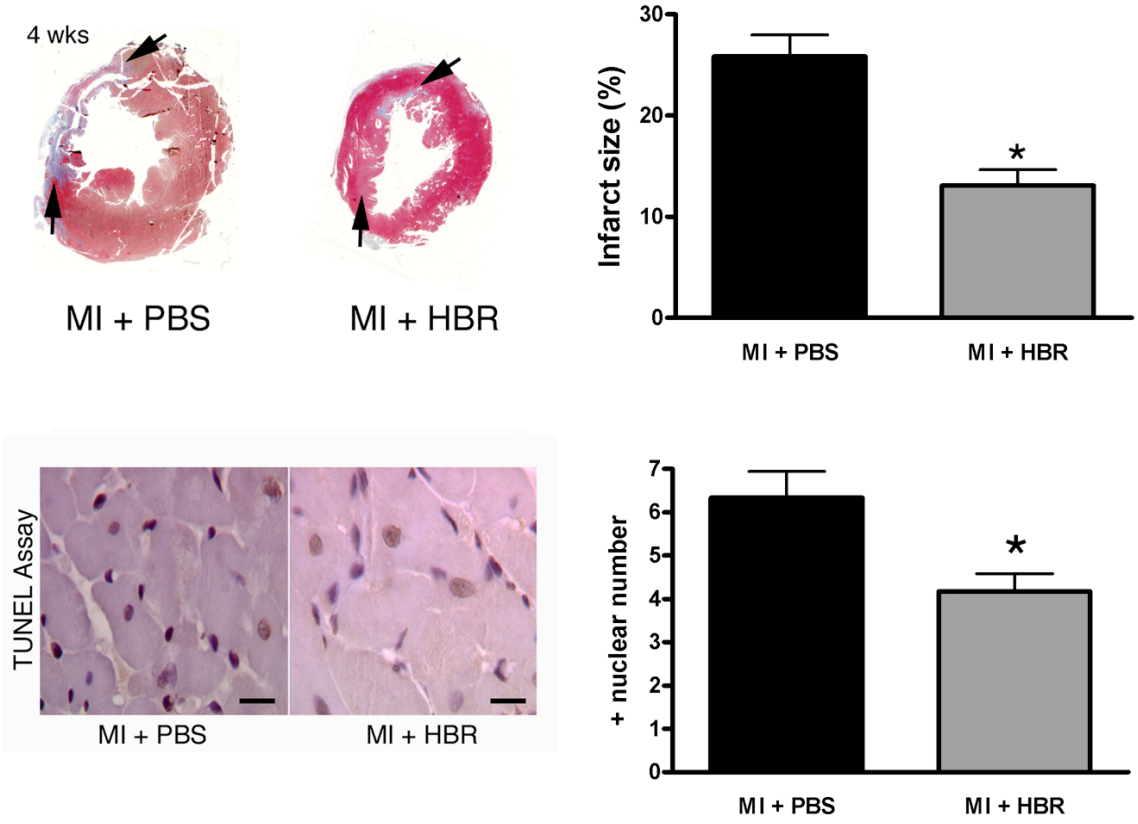

4 wks
MI + PBS
MI + HBR
Infarct size (%)
30
20
10
0
*
MI + PBS
MI + HBR
TUNEL Assay
MI + PBS
MI + HBR
+ nuclear number
7
6
5
4
3
2
1
0
*
MI + PBS
MI + HBR

Figure 1B

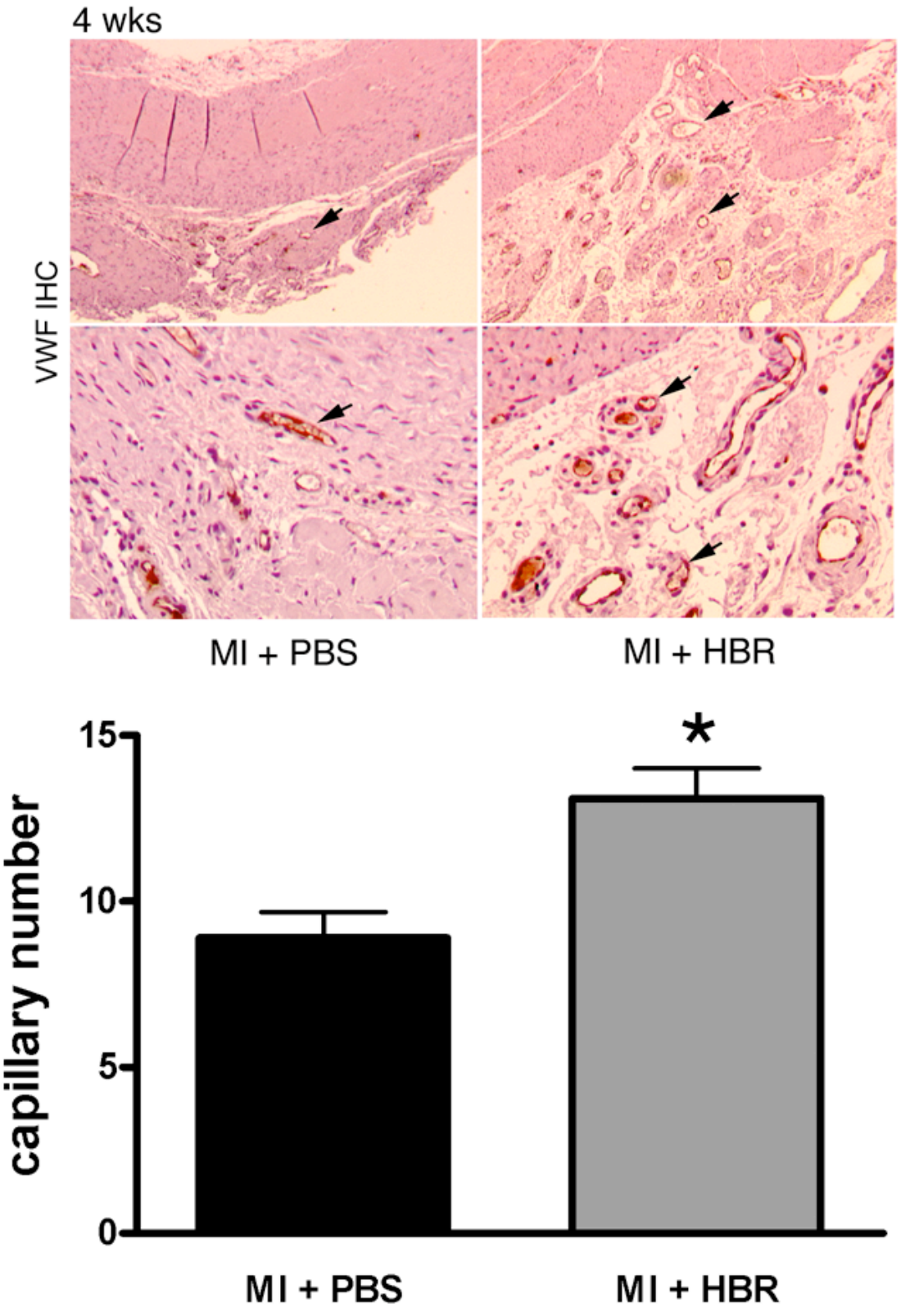

4 wks
VWF IHC
MI + PBS
MI + HBR
capillary number
15
10
5
0
*
MI + PBS
MI + HBR

Figure 1C

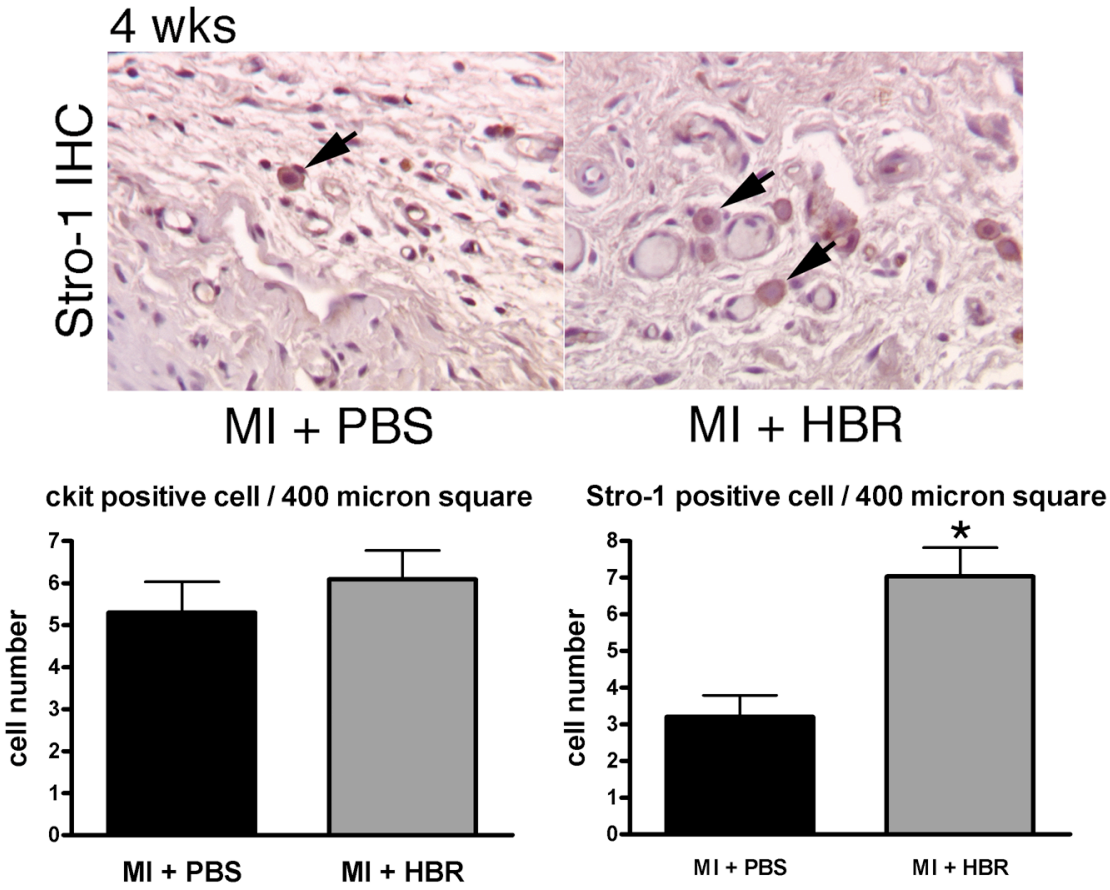

4 wks
Stro-1 IHC
MI + PBS
MI + HBR
ckit positive cell / 400 micron square
cell number
0
1
2
3
4
5
6
7
MI + PBS
MI + HBR
Stro-1 positive cell / 400 micron square
cell number
0
1
2
3
4
5
6
7
8
*
MI + PBS
MI + HBR

Figure 1D

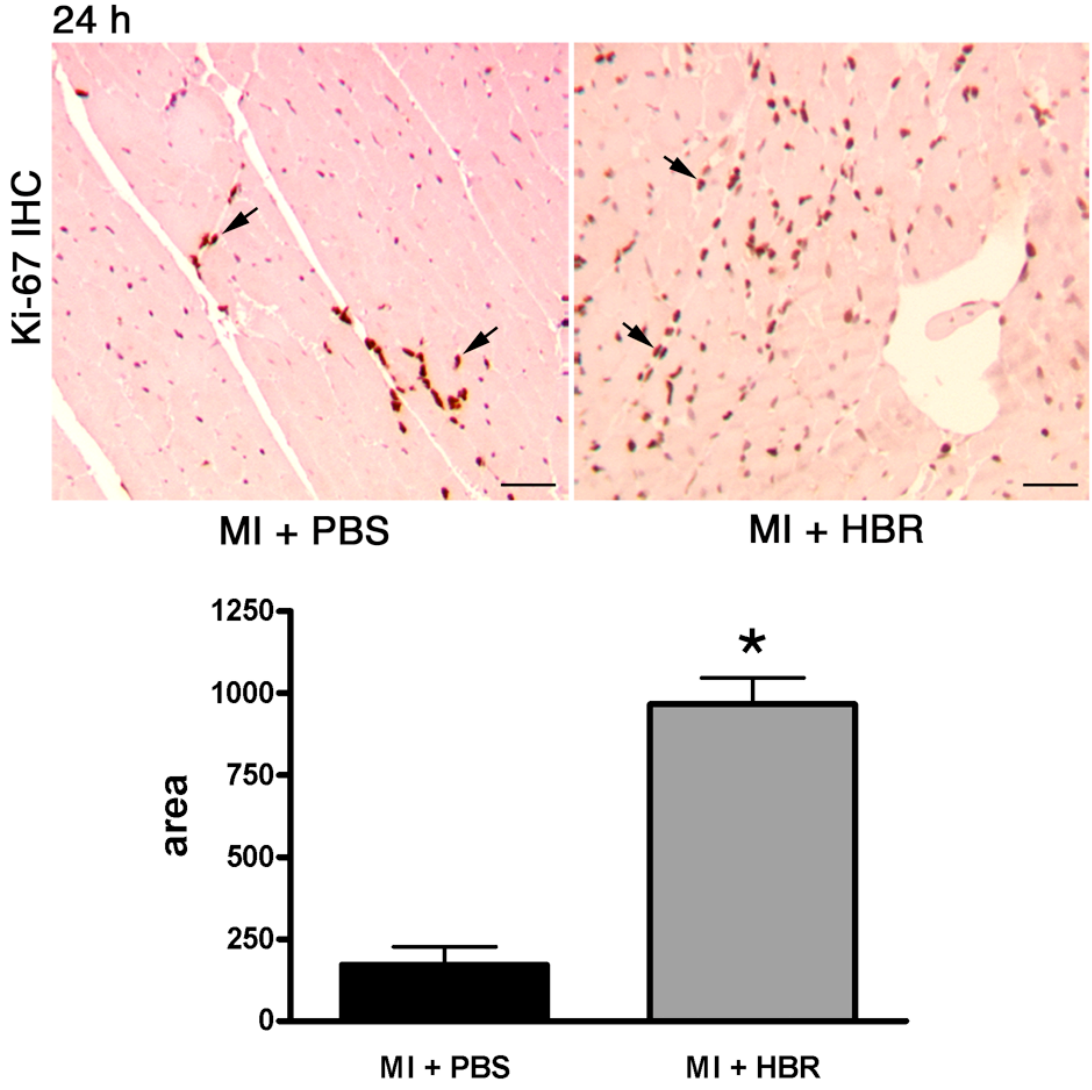

24 h
Ki-67 IHC
MI + PBS
MI + HBR
1250
1000
750
500
250
0
area
*
MI + PBS
MI + HBR

Figure 1E

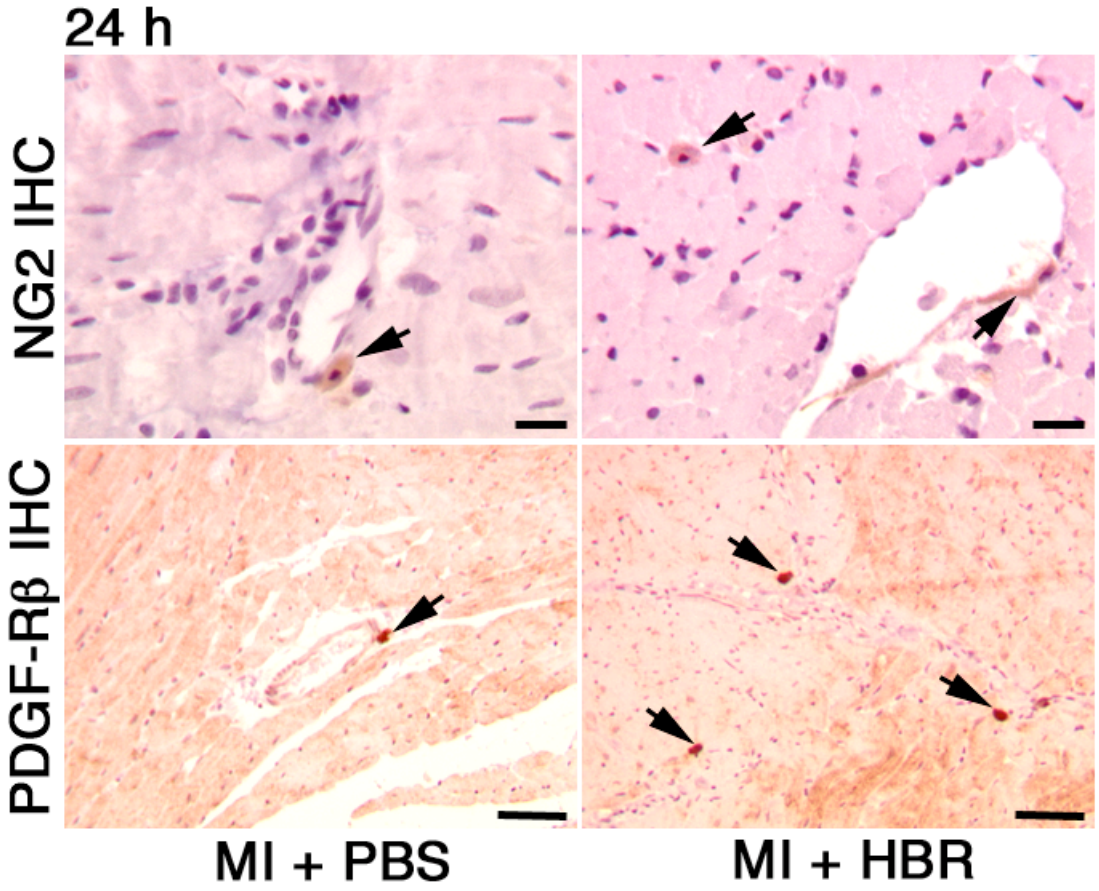
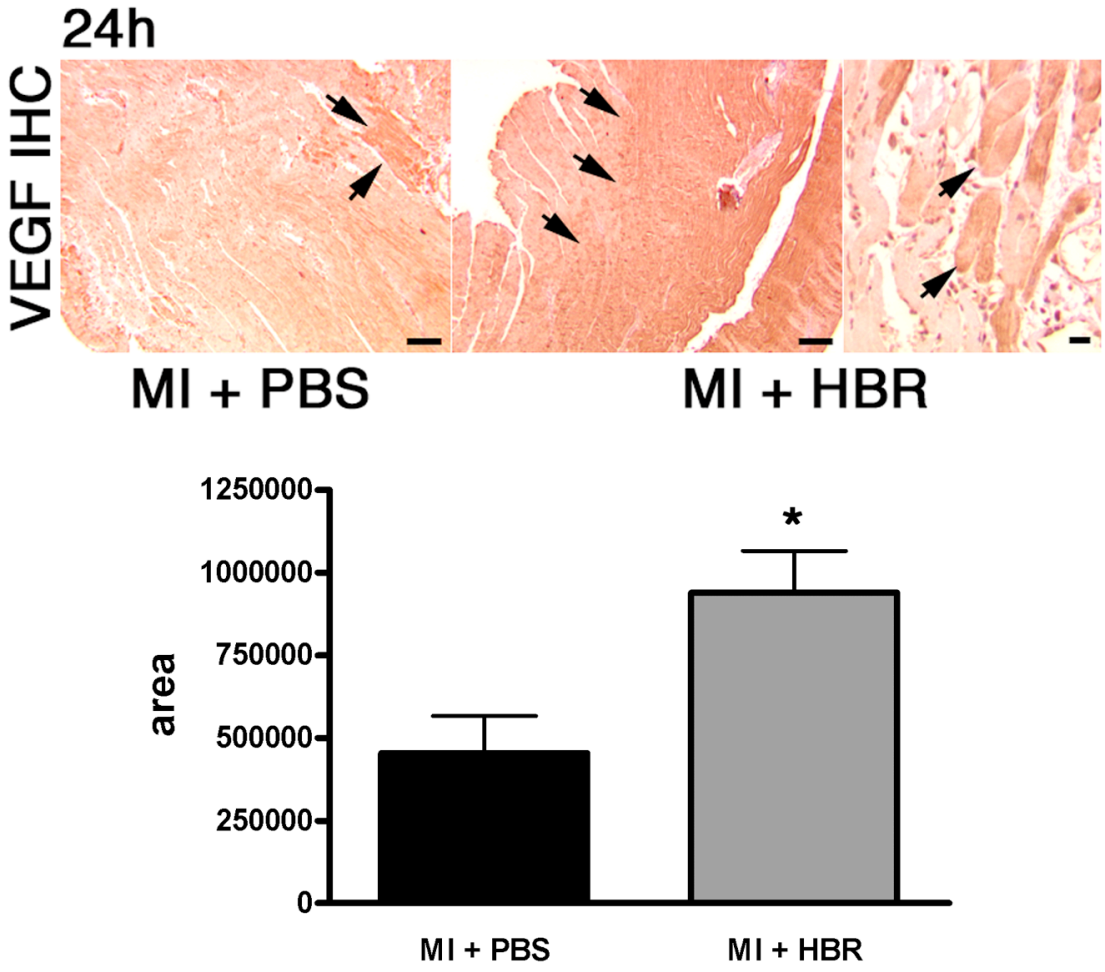


Figure 1F


24h
VEGF IHC
MI + PBS
MI + HBR
1250000
1000000
750000
500000
250000
0
area
*
MI + PBS
MI + HBR