

# Serum Collected during and after Fetal Sheep Bypass Stimulates Nitric Oxide and Endothelin-1 Production by Umbilical Vein Endothelial Cells

Hani Siddeek; Pirooz Eghtesady, MD, PhD; Kenneth E. Clark, PhD; Danielle J. Everman; Connie J. Wagner, BS; Jodie Y. Duffy, PhD

Cincinnati Children's Hospital Medical Center, Division of Cardiothoracic Surgery, Cincinnati, Ohio

University of Cincinnati College of Medicine, Departments of Surgery, Obstetrics and Gynecology, Cincinnati, Ohio

## Abstract

**Objective:** Placental dysfunction is a key barrier to successful fetal bypass for repair of congenital heart defects *in utero*. Endothelial cells regulate vascular tone during fetal bypass through interactions of vasodilation by nitric oxide (NO) and endothelin-1 (ET-1)-mediated vasoconstriction. The objective was to determine the time during fetal bypass when endothelial cell-mediated changes occur.

**Methods:** Human umbilical vein endothelial cells (HUVEC) were cultured in media containing 10% serum collected from ovine fetuses (n=3) that underwent 30 min of bypass, then were maintained for 120 min. Serum was collected before bypass, from pump prime before initiation of bypass, 30 min on bypass, or 30 and 120 min after fetal bypass. Control cells were cultured in normal fetal sheep serum. Cells were harvested 24 and 48 hr after addition of fetal serum. NO production was measured in real time with an electrochemical detection system (inNO-T, Harvard Apparatus). ET-1 was measured in the culture media by ELISA.

**Results:** NO production by HUVEC after 24 and 48 hr was stimulated above control levels by fetal serum collected during and up to 120 min after fetal bypass (p<0.05). Serum collected from fetuses that were surgically instrumented, but not yet subjected to bypass, decreased NO levels below controls (p<0.05). Stimulation of ET-1 after 24 and 48 hr of HUVEC culture peaked with serum collected at 30 min after fetal bypass (p<0.05 compared with control), but was elevated above control levels at each collection time point (p<0.05).

**Conclusions:** Fetal bypass releases serum proteins that elevate endothelial cell NO and ET-1 production during and for at least 120 min after bypass. Although the specific regulatory proteins remain to be identified, the NO and ET-1 pathways share circulating mediators and participate in a feedback loop to modulate vascular tone.

## Introduction

Placental dysfunction is a key barrier to successful fetal bypass for repair of congenital heart defects *in utero*. During neonatal and adult bypass, endothelial cells produce the peptide endothelin-1 (ET-1), a potent vasoconstrictor and mitogenic agent for smooth muscle cells, and nitric oxide (NO), a vasodilator that relaxes vessels and reduces hypoxia. Endothelial cells regulate vascular tone during bypass through interactions of vasodilation by NO and ET-1-mediated vasoconstriction. Additionally, ET-1 and eNOS gene expression are regulated by the IκBα/NF-κB pathway. The purpose of the experiment is to determine endothelial production of ET-1 and NO at various stages of fetal bypass.

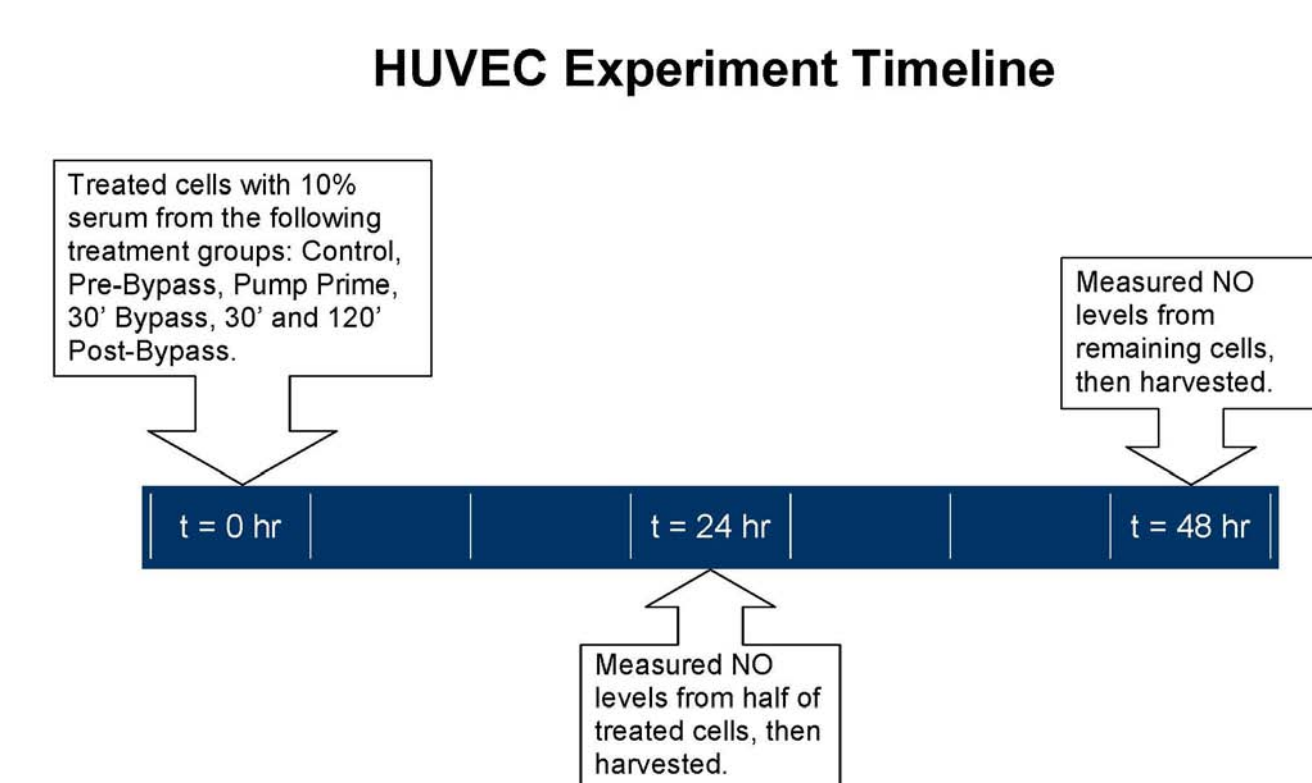


Figure 1. HUVEC Experiment Timeline.

## Aim

- ❖ To characterize changes in NO and ET-1 production by endothelial cells after 24 and 48 hours of culture in conditioned fetal sheep serum.
- ❖ To determine the time during fetal bypass when endothelial cell-mediated changes occur.
- ❖ To correlate changes in NO and ET-1 with incubation in fetal sheep serum collected throughout bypass.

## Methods

- Blood was collected from ovine fetuses (n=3) that underwent 30 minutes of bypass, then were maintained for 120 minutes.
- Serum was isolated before bypass, from the pump prime before initiation of bypass, 30 minutes on bypass, or 30 and 120 minutes after fetal bypass.
- Human umbilical vein endothelial cells (HUVEC), when 50-60% confluent, were cultured in media containing 10% fetal sheep serum and incubated at 5% carbon dioxide and 95% air. Control cells were cultured in normal fetal sheep serum.
- Cells were harvested 24 and 48 hours after addition of fetal serum.
- NO production was measured in real time with an electrochemical detection system (inNO-T, Harvard Apparatus). NO levels were measured at two areas in two cell culture plates immediately before harvesting. The mean was calculated and used for analysis.
- ET-1 production by HUVEC at 24 and 48 hours was measured in the culture media by ELISA (R&D Diagnostics).
- ET-1 assay results were read on a Multiskan EX microplate reader (Thermo EC) using Ascent software for data handling and analysis.
- Western blots were performed by SDS-PAGE then immunoblotted with antibodies for tubulin and IκBα (Santa Cruz Biotechnology, Santa Cruz, CA).
- IκBα was visualized with a chemiluminescent detection system according to the manufacturer's instructions (Invitrogen). Protein levels are reported as a ratio of IκBα to tubulin levels on the same immunoblot to correct for background effects.
- Statistical analysis was determined by student's t-test with significance at p<0.05. Data are mean ± SD.

## Results

- ❖ NO production by HUVEC after 24 and 48 hr was increased above control levels by fetal serum collected during and up to 120 min after fetal bypass (p<0.05, Figure 2).
- ❖ Serum collected from fetuses that were surgically instrumented, but not yet subjected to bypass, decreased NO levels below controls (p<0.05).
- ❖ Production of ET-1 after 48 hr of HUVEC culture was elevated above control levels at each collection time point (p<0.05, Figure 3).

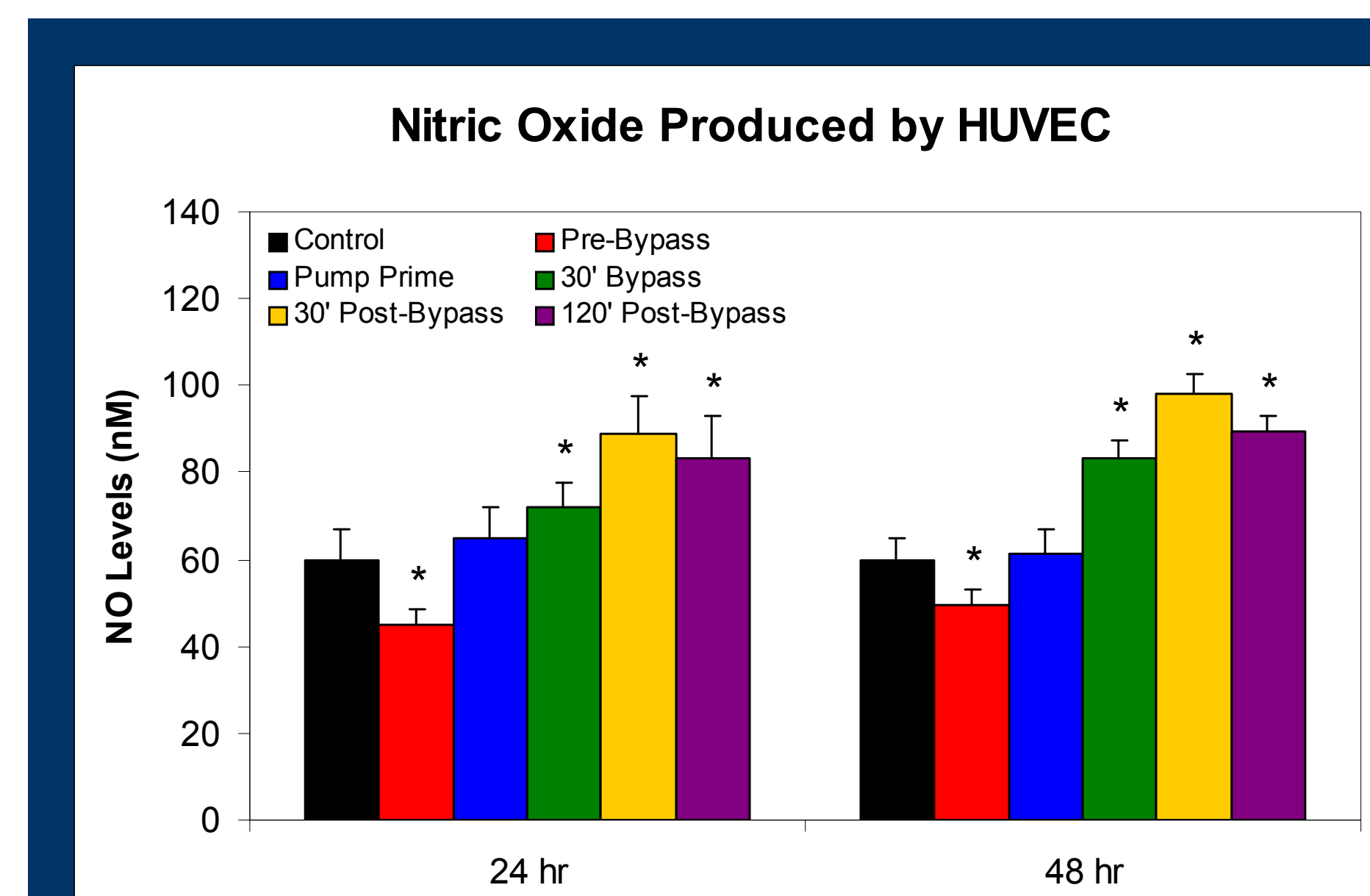


Figure 2. Nitric Oxide Produced by HUVEC. NO production by HUVEC after 24 and 48 hr was stimulated above control levels by fetal serum collected during and up to 120 min after fetal bypass (p<0.05). Serum collected from fetuses that were surgically instrumented, but not yet subjected to bypass, decreased NO levels below controls (p<0.05). n = 3 cell culture replicates, \* p<0.05 versus control at same time.

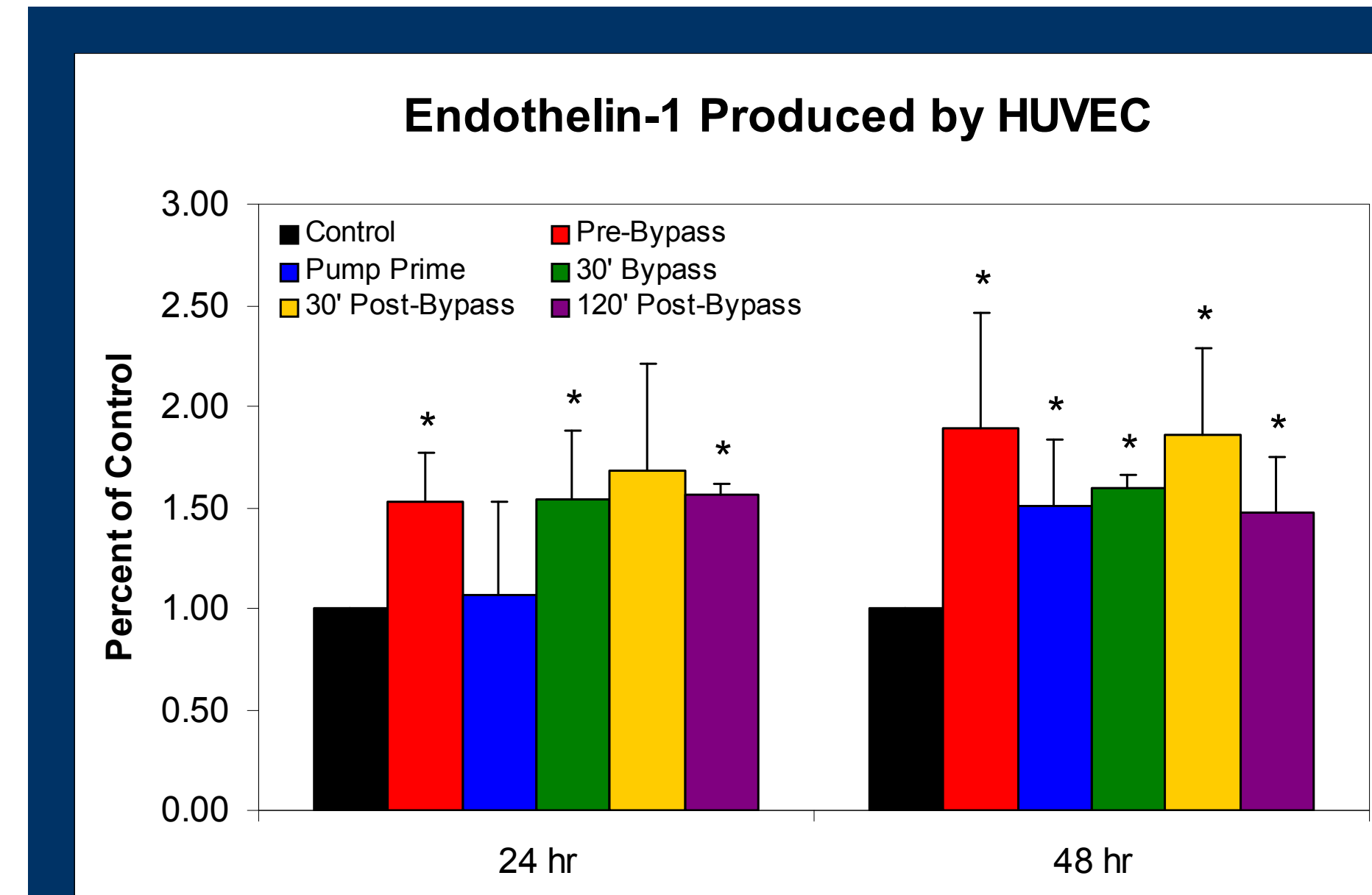


Figure 3. Endothelin-1 Produced by HUVEC. Levels of ET-1 after 24 and 48 hr were elevated above control levels at most collection time points (p<0.05), but did not differ between time points. n = 3 cell culture replicates, \* p<0.05 versus control at same time.

## Results (continued)

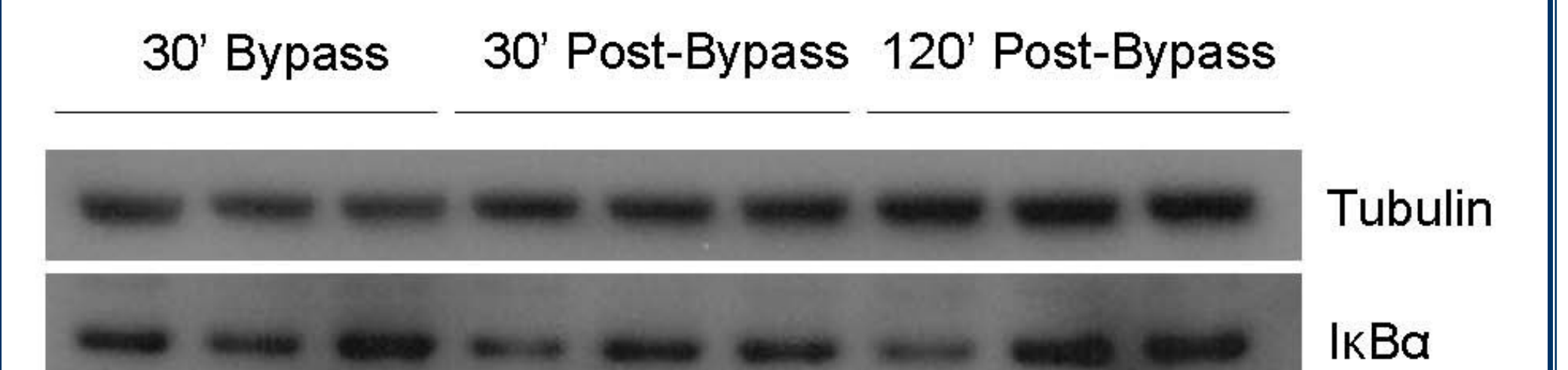


Figure 4. Representative Western Blot of IκBα and Tubulin at 48 hr of Culture.

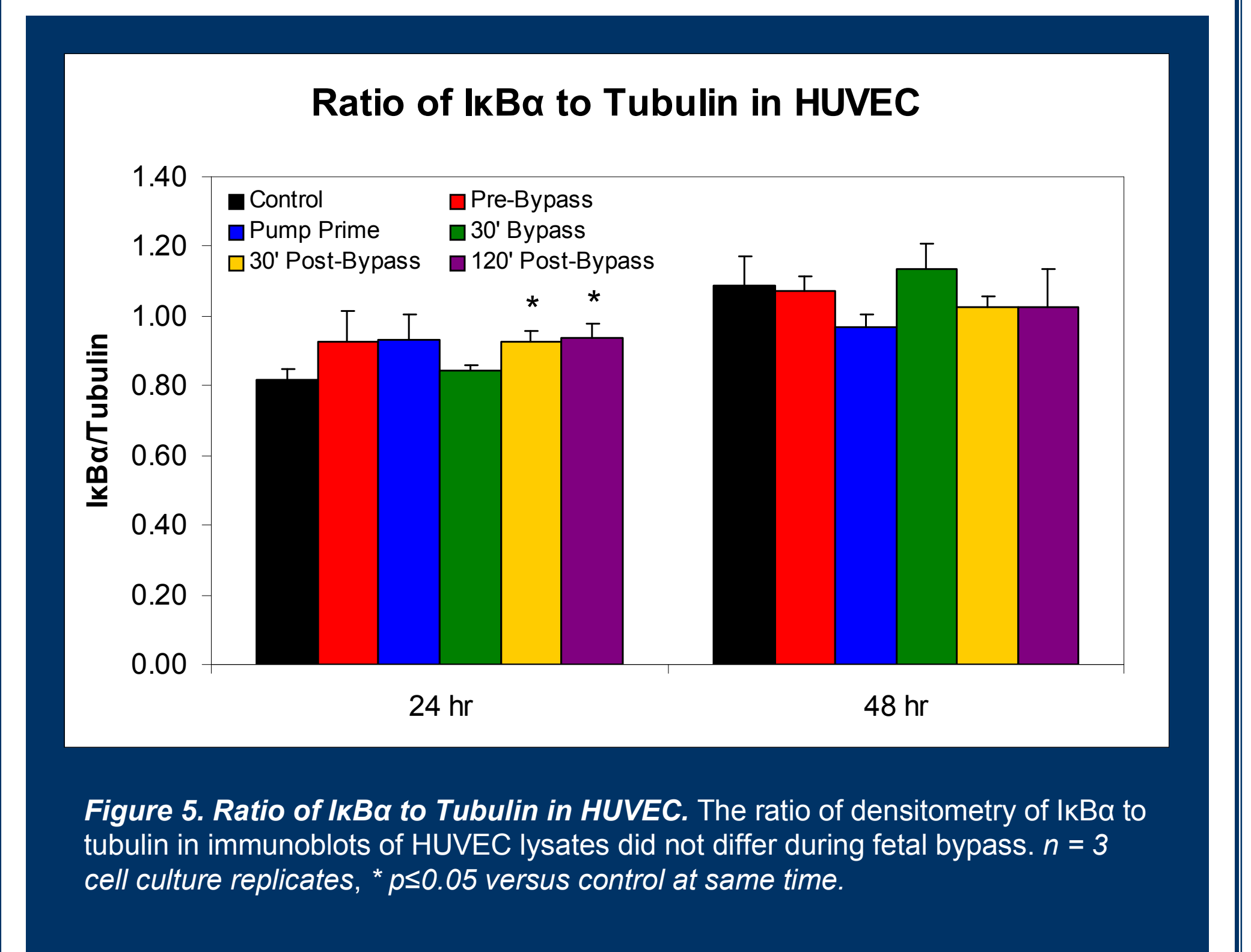


Figure 5. Ratio of IκBα to Tubulin in HUVEC. The ratio of densitometry of IκBα to tubulin in immunoblots of HUVEC lysates did not differ during fetal bypass. n = 3 cell culture replicates, \* p<0.05 versus control at same time.

## Conclusions

- Fetal cardiac surgery and bypass released serum factors that elevated endothelial cell NO and ET-1 production during and for at least 120 min after bypass.
- Serum collected before bypass decreased NO production in HUVEC at 24 hr and 48 hr of incubation.
- Although the specific regulatory mechanisms remain to be identified, the NO and ET-1 pathways share circulating mediators and might participate in a feedback loop to modulate vascular tone during fetal surgery.
- IκBα, a regulator of NF-κB activity, did not appear to regulate changes in ET-1 and eNOS production in endothelial cells cultured in fetal sheep serum collected during bypass.

No Financial or Regulatory Disclosures