Role of Oxidative Stress in Multiparity-Induced Endothelial Dysfunction

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ABSTRACT

Multiparity is associated with increased risk of cardiovascular disease. We tested whether multiparity induces oxidative stress in rat vascular tissue. Coronary arteries and thoracic aorta were isolated from multiparous and age-matched virgin rats. Relaxation to acetylcholine (ACh) and sodium nitroprusside (SNP) was measured by wire myography. We also tested the effect of the superoxide dismutase mimic MnTE2PyP (30µM), the NADPH oxidase inhibitor apocynin (10µM), and the peroxynitrite scavenger FeTPPs (10µM) on ACh-mediated relaxation in coronary arteries. Vascular superoxide anion was measured using the luminol derivative L-012, and NO generation by the Griess reaction. Multiparity reduced maximal response and sensitivity to ACh in coronary arteries (E_max: multiparous 49%±3 vs. virgins 95%±3; EC50: multiparous 135±1nM vs. virgins 60±1nM), and in aortic rings (E_max: multiparous 38%±3 vs. virgins 79%±4; EC50: multiparous 160±2nM vs. virgins 90±3nM). Coronary arteries from the two groups relaxed similarly to SNP. Superoxide anion formation was significantly higher in both coronary arteries (2.8-fold increase) and aorta (4.1-fold increase) from multiparous rats compared with virgins. In multiparous rats, incubation with MnTE2PyP, apocynin, and FeTPPs improved maximal relaxation to ACh (MnTE2PyP: 74±5%; vehicle: 41±5%; Apocynin: 73±3% vs. vehicle: 41±3%; FeTPPs: 72±3% vs. vehicle: 46±3%) and increased sensitivity (EC50: MnTE2PyP: 61±0.5nM vs. vehicle: 91±1nM; Apocynin: 45±3nM vs. vehicle: 91±6nM; FeTPP: 131±2nM vs. vehicle: 185±1nM). Multiparity also reduced total nitrate/nitrite levels (multiparous: 2.5±2µmole/mg protein vs. virgins: 7±1µmole/mg protein), and eNOS protein levels (multiparous: 0.53±0.1protein/actin vs. virgins: 1.0±0.14protein/actin). These data suggest that multiparity induces endothelial dysfunction through decreased nitric oxide bioavailability and increased ROS formation.
Normal pregnancy is a physiological condition characterized by an increase the production of reactive oxygen species (11,16). However, it is not clear whether, in the long-term, this oxidative burden contributes to cardiovascular diseases in later life. Epidemiological studies have shown an increase in cardiovascular morbidity and mortality among post-menopausal women with ≥ 4 children (14,18). In addition, Lawlor et al found multiparous women to have a higher risk for coronary heart diseases, which was associated with an adverse lipid profile and diabetes [Lawlor, Emberson, et al. 2003 #13208]. Our previous studies showed that multiparity increased the contractile sensitivity to phenylephrine in rat isolated mesenteric arteries, and that this difference was endothelium-dependent. Incubation with the nitric oxide synthase inhibitor, L-NAME caused a significant increase in phenylephrine sensitivity in mesenteric arteries from virgin rat, but not in vessels from multiparous animals. These data demonstrate that repeated pregnancy blunts the activity of the vasodilatory NO system. This suggests that multiparity might cause endothelium damage, which could ultimately cause endothelium dysfunction (7). We proposed that the recurrent periods of oxidative stress associated with pregnancy contribute to endothelial dysfunction and then cardiovascular events much later in life.

Oxidative stress occurs when the production of ROS, including free radicals such as O$_2^\cdot$\textsuperscript{−}, H$_2$O$_2$, and \textsuperscript{'OH}, exceeds the handling capability of intracellular antioxidant enzymes and extracellular antioxidant defenses (3). Oxidative stress plays a major role in the development of endothelial dysfunction (33). Endothelial dysfunction has been shown to be at least partly dependent on the production of reactive oxygen species (ROS), and the subsequent decrease in vascular bioavailability of nitric oxide (NO) (10).

One important mechanism underlying endothelial dysfunction is inactivation of endothelium derived nitric oxide (NO) by superoxide anion (O$_2^\cdot$\textsuperscript{−}). Nitric oxide (NO) reacts with
O$_2^-$ to form peroxynitrite (ONOO$^-$) (2). Unlike NO, which readily activates guanylyl cyclase and increases cyclic GMP formation in vascular smooth muscle, ONOO$^-$ is a much weaker stimulus for guanylyl cyclase (31). Thus, any reaction of NO with O$_2^-$ impairs NO-induced relaxation.

Peroxynitrite may also alter other enzyme systems that are important in vascular homeostasis. For example, ONOO$^-$ causes tyrosine nitration of various proteins including prostacyclin synthase leading to inactivation of the enzyme (37). Also, ONOO$^-$ oxidizes both tetrahydrobiopterin (24) and the zinc thiolate cluster of eNOS (36), processes that may limits eNOS derived NO production, and preferentially increases O$_2^-$ over NO production (21).

Increased ROS/ONOO$^-$ production may also affect vascular homeostasis by indirect mechanisms. A large body of evidence shows that increased oxidative stress may promote atherosclerotic disease through the oxidation of low density lipoproteins (LDL), adhesion of leukocytes to the endothelium and migration of leukocytes into the subendothelial space (35).

In this study we analyzed the potential role of oxidative stress as a mechanism underlying endothelial dysfunction in the multiparous rat. Our data show a decrease in endothelium-dependent relaxation in coronary vessels and aortic rings from multiparous animals. This was accompanied by increased superoxide anion formation. In addition, multiparity significantly reduced vascular eNOS expression. Our findings suggest that multiparity induces endothelial dysfunction through increased ROS and decreased eNOS expression, which leads to reduced NO bioavailability.
MATERIAL AND METHODS

Animals and housing: Eight to nine-month-old female Long Evans multiparous rats were obtained from Charles River (St. Foy, Quebec, Canada). These animals had undergone five pregnancies; their age at first pregnancy was 56 days. Control aged-matched virgin rats were raised in the same facility as the multiparous. A period of at least 1 month from arrival was allowed to elapse before the experiments were started, during which time, the rats were held in the University of Alberta animal facility on a 12:12-h light-dark cycle, in a humidity and temperature-controlled environment. To prevent obesity, they were restricted to just three pellets (20 g) of food per day (17).

Preparation of rat coronary arteries and aortic rings: Rats were anesthetized with sodium pentobarbital (60 mg/kg, IP). A thoracotomy was performed; heart and thoracic aorta were quickly excised and placed in ice-cold oxygenated Krebs-Henseleit buffer. After the right ventricle and anterior wall of the left ventricle were removed under a stereomicroscope, intramyocardial second order branches of the septal arteries was dissected from the septum facing the right ventricular cavity. Arterial segments (~2 mm long) were gently excised, transferred to the chamber of a small vessel myograph (Kent Scientific, Litchfield, CA, USA) containing 5 ml buffer, and mounted onto tungsten wires (diameter 40 µm). The arteries were allowed to equilibrate in oxygenated (95% O₂ and 5% CO₂) Krebs-Henseleit buffer. The composition of the buffer is (in mM) 118.3 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, and 11.0 glucose. Vessels were allowed to stabilize for 30 min in the Krebs-Henseleit buffer under zero tension, during which time the buffer solution was changed at 10 min intervals. This was
followed by a preconditioning stretch of 0.2 g tension, after which vessels were rested at 0.2 g tension and allowed to stabilize in Krebs-Henseleit buffer for a further 30 min. The vessels were then radially stretched to their optimal lumen diameter for active tension development, i.e. to an internal circumference equal to 90% of that achieved in vessels exposed to a passive tension equivalent to a transmural pressure of 100 mmHg. Our preliminary experiments on rat coronary arteries indicated that the optimal active tension is 0.2 grams.

The thoracic aorta was gently excised. Fat and connective tissue were removed, and the aorta was cut transversely into rings of 2-3 mm in width. The rings were mounted between two stainless steel wire hooks with extreme care being taken to avoid damaging the endothelium. They were then transferred to the chamber of a myograph (Kent Scientific, Litchfield, CA, USA) containing Krebs-Henseleit solution continuously gassed with 95% O₂-5% CO₂ (37°C, pH 7.4). Aortic rings were allowed to equilibrate for 60 min at an initial resting tension of 1 g, and the bathing solution was changed every 10 min according to the previously described protocol. Isometric force for all experiments was recorded online (Windaq data acquisition software).

**Vascular Reactivity:** After the equilibration period, the responsiveness of each individual coronary artery segment or aortic ring was confirmed by its vasoconstrictive response to KCl. The integrity of the vascular endothelium was confirmed pharmacologically by acetylcholine-induced relaxation of arteries which had been preconstricted with U46619 (thromboxane A₂ receptor agonist). Tissues which did not elicit a reproducible and stable contraction with U46619 were discarded from the study. Preparations were then washed three times with Krebs-Henseleit buffer and allowed to relax fully for 30 min before the experimental protocol began.

The arteries were constricted with U46619 at a submaximal dose of 1 µM (EC₈₀). After
reaching a plateau of contraction, cumulative concentration response curves to acetylcholine (ACh, 0.1 nM – 100 µM) and sodium nitroprusside (SNP, 0.1 nM – 100 µM) were obtained to evaluate endothelium-dependent and independent relaxations, respectively. In addition, we measured endothelium-dependent relaxation of rat aortic rings by performing cumulative concentration-response curves to ACh (0.1 nM – 100 µM).

In a second series of experiments, ACh-induced vasodilation in rat coronary arteries was measured after 30 min incubation with one of the following: the cell permeable superoxide dismutase mimetic MnTE2PyP (30 µM) (6); the NADPH oxidase inhibitor apocynin (10 µM) (32); the ONOO\(^-\) scavenger FeTPPs (10 µM) (9); or the NOS inhibitor L-NAME (30 µM). In all cases, the responses were compared with those obtained using the same vessel prior to incubation with the test substance (control). The vasorelaxant responses are expressed as percent decrease from U46619-induced contraction i.e. the amount of contraction produced by 1 µM U46619 in each vessel from its initial resting tension was considered to be 100%.

**Superoxide anion assays:** Superoxide anion (O\(_2^{-}\)) generation in isolated coronary vessels and aorta was measured using a chemiluminescence assay. Vascular segments from each rat (virgin and multiparous) were incubated in Krebs-Henseleit buffer containing 500 µM of the highly sensitive luminol derivative L-012 (Wako Pure Chemical Industries, Osaka, Japan) (32). Chemiluminescence was measured in coronary arteries using the TD-20/20 Luminometer (Turner Designs Sunnyvale CA, USA), and in aortic tissue using the FB12 Luminometer (Berthold Detection System TN, USA).

**Tissues nitrate/nitrite level:** For analysis of nitric oxide (NO) formation, we evaluated
vascular homogenate NO by measuring total nitrate and nitrite levels using the Griess reaction method (Cayman chemical, MI USA). Although we measured vascular reactivity in both coronary arteries and aorta, tissue nitrate/nitrite levels were measured only in the aorta due to the difficulty of obtaining sufficient tissue from the coronary arteries.

Briefly, aortic homogenate was incubated with nitrate reductase enzyme (10 mU) and NADPH (cofactor) for 3 hours at 37°C. The total nitrite in each sample was then determined by adding sulfanilamide (Griess reagent 1), followed by N-(1-naphthyl) ethylenediamine (Griess reagent 2). The mixture was incubated at 37°C for 10 minutes. Absorbance at 540 nm was recorded and the concentrations of NO₂ were calculated from a standard curve constructed using NaNO₂ and NaNO₃ standards. Nitrate/nitrite levels were expressed as µM/mg tissue protein.

**Western blot:** For analysis of eNOS protein, frozen rat aortic arteries were crushed using a mortar and pestle that was cooled with dry ice. Again, we used aortic tissues homogenates to measure eNOS protein, due to the small size of coronary arteries and the difficulty of obtaining sufficient tissue. The resulting powder was diluted 1:4 w/v in 50mM Tris-HCl (pH 7.4) buffer containing 3.1mM sucrose, 1mM dithiothreitol, 10 mg/ml leupeptin, 10 mg/ml soybean trypsin inhibitor, 2 mg/ml aprotinin and 0.1% Triton X-100. This solution was then homogenized on ice using a microcentrifuge tube pestle. The homogenate was centrifuged at 10,000 g for 5 min at 4°C and the supernatant was kept on ice.

Protein concentration was measured using the BCA protein assay (Pierce, IL USA). Aortic homogenate samples (40 µg) were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, probed with monoclonal antibody against eNOS (Cell Signaling, Boston, MA) followed by secondary
antibody and enhanced chemiluminescence for detection (Amersham Pharmacia, San Francisco, CA). The membrane was stripped and re-probed for β-actin content using a species monoclonal antibody to verify equal loading and results were analyzed using densitometry and Image J software.

**Drugs:** Acetylcholine chloride (ACh), Sodium Nitroprusside (SNP), apocynin, and L-NAME were purchased from Sigma (St. Louis, MO). MnTE2PyP, U46619 and FeTPPs were purchased from Calbiochem (San Diego, CA USA). L-012 was purchased from Wako Pure Chemical Industries (Richmond, VA USA). L-NAME, ACh, MnTE2PyP, SNP, and FeTPPs (stock 10 mM) solutions were made in deionized water. Apocynin was dissolved in DMSO. U46619 was supplied as liquid in methyl-acetate and evaporated to dryness under a stream of nitrogen and dissolved in DMSO to stock solution (10 mM). Desired concentration of SNP, apocynin, and U46619 were made in deionized water. L012 was dissolved in deionized water to a final concentration of 20 mM and then diluted into Krebs-Henseleit buffer to the assay concentration 500 µM.

**Data analysis:** All values are shown as mean ± SE mean. Maximal relaxation (E_max) and half-maximal effective dose (EC_{50}) were calculated from individual dose-response curves. EC_{50} values were derived using Graph-Pad Prism. Statistical comparisons between these parameters for the virgin and multiparous groups were analyzed by one-way analysis of variance with Bonferroni's test for comparison between all pairs of groups. In some experiments, statistical differences were determined by Student's t test. The results were considered significant when p < 0.05.
RESULTS

Effect of multiparity on ACh- and SNP-mediated coronary arteries and aortic rings

Vasorelaxation: Concentration-response curves to U46619 were constructed for coronary arteries and aortic rings from multiparous and virgin rats. U46619 produced maximal contraction in both groups at a concentration of 10 µM ($E_{max}$) ($n=6$, data not shown). A submaximal concentration (EC$_{80}$, 1 µM) was chosen for preconstriction in subsequent experiments; the constriction to this concentration of U46619 was thus defined as 100% in the relaxation curves. There was no significant difference between the two groups (multiparous and virgins) with respect to the responses to KCl (40, 60, and 80 mM, $n=4$, data not shown).

The reproducibility of the responses induced by ACh on the coronary arteries was assessed in a subset of ACh concentration-response experiments. Each vessel was exposed to the same ACh concentrations twice, separated by at 30 min of drug-free incubation. Our results produced quantitatively similar and reproducible effects on coronary arteries with repeated exposure ($n=5$, data not shown). Thus it is evident that ACh-induced coronary vascular responses in our rat model do not exhibit tachyphylaxis with successive drug application.

ACh produced concentration-dependent relaxation in coronary arteries from both virgin and multiparous rats. Maximal relaxation ($E_{max}$) was higher in coronary arteries from virgin (95% ± 3, n=9) than from multiparous rats (49% ± 3, n=9, P<0.05) (Figure 1A). There was also a rightward shift in the response of coronary arteries from multiparous rats (EC$_{50}$ = 135 ± 1 nM) compared with the response from virgin rats (EC$_{50}$ = 60 ± 1 nM, P<0.05). L-NAME completely blocked ACh-mediated relaxation in vessels from both virgin and multiparous animals (Figure 1A). Coronary arteries from multiparous and virgin rats responded similarly to the NO donor
SNP (Figure 1B). Aortic rings from multiparous rats exhibited decreased $E_{\text{max}}$ to ACh (38% ± 3, $P<0.05$) compared to virgin rats (79% ± 4) (n = 5 in both groups); and a rightward shift in the concentration-response curve ($EC_{50}$ multiparous: 160 ± 2 nM vs. $EC_{50}$ virgins: 100 ± 3 nM) (Figure 2).

**Effect of SOD mimetic (MnTE2PyP) on endothelium-dependent relaxation of rat coronary arteries:** Incubation of coronary vessels from multiparous rats with MnTE2PyP significantly improved maximal relaxation ($E_{\text{max}}$ MnTE2PyP = 74% ± 5, n=6 vs. Vehicle = 41% ± 5, n=6, $P<0.05$) and sensitivity ($EC_{50}$: MnTE2PyP = 61 ± 0.5 nM vs. Vehicle = 91 ± 6 nM) to ACh (Figure 3A). The submaximal contraction obtained at 1 µM of U46619 was not altered by MnTE2PyP. Nor did MnTE2PyP alter ACh-mediated relaxation in coronary arteries from virgin rats (data not shown).

**Effect of the ONOO$^-$ scavenger (FeTPPs) on endothelium-dependent relaxation of rat coronary arteries:** Incubation of coronary vessels from multiparous rats with FeTPPs significantly improved maximal relaxation ($E_{\text{max}}$ FeTPPs = 72% ± 3, n=7 vs. Vehicle = 46% ± 3, n=7) and sensitivity ($EC_{50}$: MnTE2PyP = 61 ± 0.5 nM vs. Vehicle = 91 ± 6 nM) to ACh (Figure 3B). The submaximal contraction obtained at 1 µM of U46619 was not altered by FeTPPs. Nor did FeTPPs alter ACh-mediated relaxation in coronary arteries from virgin rats (data not shown).

**Effect of the NADPH oxidase inhibitor apocynin on endothelium-dependent relaxation of rat coronary arteries:** Incubation of coronary vessels from multiparous rats with apocynin significantly improved maximal relaxation (Apocynin = 73±3% vs. Vehicle = 41±3%) and
sensitivity (EC$_{50}$ apocynin: 45 ± 3 nM; EC$_{50}$ Vehicle: 91 ± 6 nM) to ACh (Figure 3C). The submaximal contraction obtained at 1 µM of U46619 was not altered by apocynin. Nor did apocynin alter ACh-mediated relaxation in coronary arteries from virgin rats (data not shown).

**Effect of multiparity on ROS levels in rat coronary arteries:**
To evaluate the contribution of O$_2^-$ in multiparity-reduce acetylcholine mediated relaxation, we assessed the effect of multiparity on O$_2^-$ formation using chemiluminescence. Superoxide anion production was significantly higher in both coronary vessels and aortic tissue from multiparous compared to virgin rats (Figure 4A,B).

**Effect of multiparity on NO production in rat aorta:**
To test whether the increased O$_2^-$ formation in vascular tissues from multiparous rats reduces NO availability, we measured nitrate/nitrite using the Griess reaction. Nitrate/nitrite levels were significantly lower in aortic tissues from multiparous rats compared with those from virgins (Figure 5).

**Effect of multiparity on endothelial nitric oxide synthase levels in rat aorta:**
To establish whether there are other, non ROS-mediated, mechanisms by which multiparity might reduce endothelial-dependent relaxation, we measured eNOS protein level. eNOS protein was significantly lower in aortic tissue from multiparous rats compared with that in tissue from virgins (Figure 6A, B).
DISCUSSION

Circulating markers of oxidative stress increase during pregnancy (23,26,34). However, the question as to whether this returns to normal after parturition, and whether this might contribute to the increased risk for cardiovascular disease observed in older multiparous women (7,8,13,18,22,27,28) had not previously been addressed. Our data demonstrate that indeed, there is increased oxidative stress in both coronary arteries and aorta from multiparous rats, and that this does contribute to compromised vasodilation in both the coronary and aortic vasculature.

An imbalance between the production of ROS and NO in the vessel wall is considered to play an important role in the development of endothelial dysfunction. Superoxide anion reacts with NO to form ONOO\(^{-}\) (1). ONOO\(^{-}\) can oxidize the NOS co-factor tetrahydrobiopterin (24), and also reduces cellular transport of L-arginine, the eNOS substrate for NO production (29). It is believed by many that these events contribute to increasing O\(_2\)\(^{\cdot}\) production over NO production (24). Ultimately, endothelial dysfunction reflects the inability of the endothelium to generate adequate amounts of NO (or reduced bioavailability of NO) and to produce NO-mediated vasorelaxation.

Our data show that coronary arteries and aortic rings from multiparous rats exhibit significantly decreased NO-dependent relaxation in response to ACh. That is, repeated pregnancy causes a long-term reduction in endothelium-dependent vasorelaxation. This finding is consistent with our previous studies showing that the enhanced vasoconstriction to PE of mesenteric arteries from multiparous rats is also NO-dependent (7). We have also shown that the decreased NO-dependent relaxation in arteries from multiparous rats was associated with increased O\(_2\)\(^{\cdot}\) levels as shown by chemiluminescence. These data are in agreement with reports
of decreased NO-dependent relaxation in association with other oxidative stress conditions such as diabetes (32), smoking (15) and hypertension (20).

We evaluated endothelium-independent relaxation in rat coronary arteries by performing concentration-response curves to the NO donor SNP. Our results showed that coronary arteries from both multiparous and virgin rats relaxed similarly to SNP. These data confirm that, in our model, multiparity caused impairment only of endothelium-dependent relaxation i.e. that the vascular smooth muscle response to NO was normal. Furthermore, L-NAME blocked ACh-mediated relaxation in the coronary arteries. These data confirm that, under the conditions used in these experiments, ACh mediated relaxation was mediated through endothelium-derived NO, and that the reduction in ACh-mediated relaxation in coronary arteries from multiparous rats may indeed be attributed to endothelial dysfunction.

Our finding that multiparity reduced endothelium-dependent relaxation and increased \( \cdot O_2 \) levels, prompted us to assess the effect of the SOD mimetic MnTE2PyP on ACh-mediated relaxation. Incubation of coronary vessels from multiparous rats with MnTE2PyP significantly improved ACh-mediated relaxation. To evaluate the specific contribution of \( \cdot O_2 \) in the multiparity-induced decrease in ACh-mediated relaxation, we assessed the effect of apocynin (NADPH oxidase inhibitor) on ACh-mediated relaxation. Apocynin improved ACh-mediated relaxation in coronary arteries from multiparous rats. These data suggest that multiparity decreases vascular relaxation by a mechanism involving \( \cdot O_2 \) formation and subsequent reduction in NO availability. This concept was supported by our finding that multiparity was also associated with reduced nitrate/nitrite level in multiparous rat aortic homogenates. Our results are thus in agreement with previous reports that multiparity attenuates the production of nitric oxide (NO) in the kidney (30).
An important characteristic of endothelial dysfunction is inactivation of NO by its reaction with $O_2^−$ to form $\text{ONOO}^−$ (5,12). FeTPPs is a drug which catalyzes the conversion of $\text{ONOO}^−$ into nitrate, thus preventing the oxidizing and nitrating reactivity (25). In accordance with this concept, we showed that incubation with FeTPPs improved ACh-mediated relaxation in coronary vessels from multiparous rats. This finding suggests that multiparity increases $O_2^−$ formation in vascular tissues, which then scavenges NO to form $\text{ONOO}^−$. We thus speculated that the impaired relaxation to ACh seen in coronary arteries from multiparous rats is due to increased $O_2^−/\text{ONOO}^−$ and decreased L-arginine availability to eNOS. Consequently, there is a reduction in NO bioavailability. We tested the NO level and eNOS protein in aortic tissues homogenates. We have shown that multiparity decreases endothelium-dependent relaxation in two functionally distinct vascular tissues, namely coronary arteries and the aorta. Our data suggest that this is due to ROS-mediated endothelial damage.

Impaired endothelium-dependent reactivity can also be caused by changes in eNOS. We therefore measured eNOS protein in rat aortic homogenates. Multiparity significantly reduced vascular eNOS protein levels. Although we recognize that eNOS expression does not necessarily reflect eNOS activity, it is likely that a reduced eNOS protein would be associated with impaired endothelium-dependent relaxation. A possible mechanism by which oxidative stress could reduce eNOS is via $\text{ONOO}^−$ activation of RhoA and its downstream target Rho kinase; there is evidence that Rho kinase decreases eNOS mRNA expression (19). Another possible mechanism could be through activation of P21 Ras. $\text{ONOO}^−$ has been shown to increase the activity and expression of P21Ras which, in turn, could inhibit eNOS protein expression (4).

In summary, our data show that multiparity (repeated pregnancy) reduces endothelium-dependent relaxation in rat coronary arteries and aortic rings. This effect was associated with
increased oxidative stress. Increased $O_2^-$ would enhance ONOO$^-$ formation, thus reducing eNOS expression and NO biosynthesis. We conclude that multiparity induces vascular endothelial dysfunction by facilitating the formation of reactive oxygen species, which then decrease NO production and bioavailability.
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FIGURE LEGENDS

**Figure 1.** Concentration-response curves for preconstricted coronary arteries from multiparous rats (open symbols) and age-matched virgin rats (closed symbols). (A) Response to ACh after preincubation with L-NAME (virgin: ▲; multiparous: △) or vehicle (virgin: ●; multiparous: ○); (B) Response to sodium nitroprusside (virgin: ●; multiparous: ○). Values are expressed as means ± SEM.

**Figure 2:** Concentration-response curves to ACh for preconstricted thoracic aortic rings from multiparous rats (open symbols, O) and age-matched virgin rats (closed symbols, ●). Values are expressed as means ± SEM.

**Figure 3:** Effect of (A) MnTE2PyP, (B) FeTPPs, and (C) apocynin on concentration-response to ACh of preconstricted coronary arteries. Vehicle-treated vessels from multiparous rats (O), age-matched virgin rats (●) and MnTE2PyP-, FeTPPs-, or apocynin-treated vessels (▼). Values are expressed as means ± SEM.

**Figure 4:** Effect of multiparity on O$_2^{-}$ formation by rat coronary arteries (A, n=6) and aorta (B, n=7) from virgin rats (Black bar, n=7) and age-matched multiparous rats (Open bar, n=7). Data are expressed as relative light unit (RLU)/mg wet tissue weight. Values are expressed as means ± S.E.M.; * p < 0.05 vs. control.

**Figure 5:** Effect of multiparity on nitrate/nitrite level in rat aortic tissue homogenates from
virgin rats (Black bar, n=7) and age-matched multiparous rats (Open bar, n=7). Data are expressed as μM/mg protein. Values are expressed as means ± SEM. * p < 0.05 vs. control

**Figure 6:** (A): Western blot analysis of eNOS expression in aortic arteries of virgin and multiparous rats. (B): Quantification of blots by densitometry. Values are expressed as means ± SEM, *p < 0.05 vs. control.
Figure A: Effect of ACh (log M) on % Relaxation.

Figure B: Effect of SNP (log M) on % Relaxation.
Superoxide formation (RLUx10^{-2}/mg wet tissue weight)

Virgin

Multiparous

Superoxide formation (RLU/mg wet tissue weight)
Nitrate/Nitrite (µM/mg protein)

Virgin Multiparous
eNOS protein level/actin (A.U.)

Virgins Multiparous

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