Prevention of vascular dysfunction and arterial hypertension in mice generated by assisted reproductive technologies by addition of melatonin to culture media

Emrush Rexhaj,1 Agim Pireva,1 Ariane Paoloni-Giacobino,2 Yves Allemann,1 David Cerny,1 Pierre Dessen,3 Claudio Sartori,1,3* Urs Scherrer,1,4* and Stefano F. Rimoldi1* 

1Department of Cardiology and Clinical Research, University Hospital, Bern, Switzerland; 2Department of Genetic and Laboratory Medicine and Swiss Center for Applied Human Toxicology, Geneva University Hospital, Geneva, Switzerland; 3Department of Internal Medicine, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland; and 4Facultad de Ciencias, Departamento de Biología, Universidad de Tarapacá, Arica, Chile

Submitted 2 September 2014; accepted in final form 14 August 2015

METHODS

STUDIES IN ANIMALS AND HUMANS demonstrate that adverse events during early life induce vascular dysfunction that may predispose to premature cardiovascular morbidity and mortality (1, 2, 10, 19, 24). Assisted reproductive technologies (ART) represent a recent important example of this problem, since ART induces vascular and cardiac dysfunction in children (26, 33, 34) and causes premature vascular aging and arterial hypertension that are associated with a shortened life span in mice (20). Consistent with observations in humans suggesting that decreased nitric oxide [nitrate/nitrite (NOx)] bioavailability contributes to vascular dysfunction in the offspring of ART (22), data in ART mice indicate that epigenetic alterations of the endothelial nitric oxide synthase (eNOS) gene that translate into decreased vascular eNOS expression and NOx synthesis underpin premature vascular aging and arterial hypertension (20). There is increasing evidence that suboptimal embryo culture conditions contribute to ART-induced epigenetic alterations (6, 12, 15), as evidenced, for example, by the fact that current culture media used for ART are unable to maintain genomic imprinting of in vitro embryos (15).

Melatonin, the main secretory product of the pineal gland, is also synthesized by many peripheral tissues, including the ovary, where it plays a role in the regulation of its function (14, 30) and has favorable effects on oocyte maturation (11). In sterile women with low fertilization rates during in vitro fertilization (IVF), oral melatonin administration improves the fertilization success (32). Finally, and most interestingly, melatonin, possibly by inhibiting DNA methyltransferase, may be involved in epigenetic regulation (13, 17, 28). We, therefore, tested the effects of the addition of melatonin to the cleavage and to the blastocyst development medium on vascular function, arterial blood pressure, and epigenetic alterations of the promoter of the eNOS gene in ART mice.

RESULTS

Culture media

Culture media

Growing population. Here, we found that, in mice, modification of culture media prevents assisted reproductive technologies-induced alteration of the cardiovascular phenotype. We speculate that this approach may allow preventing this problem in humans.

In humans and mice. In mice, ART-induced vascular dysfunction is related to epigenetic alteration of the endothelial nitric oxide synthase (eNOS) gene, resulting in decreased vascular eNOS expression and nitrite/nitrate synthesis. Melatonin is involved in epigenetic regulation, and its administration to sterile women improves the success rate of ART. We hypothesized that addition of melatonin to culture media may prevent ART-induced epigenetic and cardiovascular alterations in mice. We, therefore, assessed mesenteric-artery responses to acetylcholine and arterial blood pressure, together with DNA methylation of the eNOS gene promoter in vascular tissue and nitric oxide plasma concentration in 12-wk-old ART mice generated with and without addition of melatonin to culture media. As expected, acetylcholine-induced mesenteric-artery dilation was impaired (P = 0.008 vs. control) and mean arterial blood pressure increased (109.5 ± 3.8 vs. 104.0 ± 4.7 mmHg, P = 0.002, ART vs. control) in ART compared with control mice. These alterations were associated with altered DNA methylation of the eNOS gene promoter (P < 0.001 vs. control) and decreased plasma nitric oxide concentration (10.1 ± 1.1 vs. 29.5 ± 8.0 μM, P < 0.001 ART vs. control). Addition of melatonin (10−6 M) to culture media prevented eNOS demethylation (P = 0.005 vs. ART + vehicle), normalized nitric oxide plasma concentration (23.1 ± 14.6 μM, P = 0.002 vs. ART + vehicle) and mesentery-artery responsiveness to acetylcholine (P < 0.008 vs. ART + vehicle), and prevented arterial hypertension (104.6 ± 3.4 mmHg, P < 0.003 vs. ART + vehicle). These findings provide proof of principle that modification of culture media prevents ART-induced vascular dysfunction. We speculate that this approach will also allow preventing ART-induced premature atherosclerosis in humans.

NEWS & NOTEWORTHY

Assisted reproductive technologies-induced cardiovascular dysfunction is a major problem with possibly dramatic consequences on future morbidity and mortality in this rapidly growing population. Here, we found that, in mice, modification of culture media prevents assisted reproductive technologies-induced alteration of the cardiovascular phenotype. We speculate that this approach may allow preventing this problem in humans.
H1152  PREVENTION OF VASCULAR DYSFUNCTION IN ART MICE

IVF and embryo culture. IVF and embryo culture were done as described previously (20). Briefly, 8- to 12-wk-old female FVB mice were superovulated by intraperitoneal (IP) injection of 5 IU (0.1 ml) pregnant mare serum gonadotropin (Intervet, Zürich, Switzerland), followed 50 h later by an IP injection of 5 IU (0.1 ml) of human chorionic gonadotropin (hCG; Intervet). Fourteen hours post-hCG, cumulus-oocyte complexes were recovered from oviducts in human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA), supplemented with 5 mg/ml of human serum albumin (HAS; Irvine Scientific). Spermatozoa were collected from the cauda epididymis of 10- to 14-wk-old FVB mice and capacitated for 60 min in HTF/HSA medium at 37°C under a humidified atmosphere of 6% CO₂ in air. Oocytes were inseminated 14 h post-hCG with 10⁶ spermatozoa in HTF/HSA medium for 4 h at 37°C and 6% CO₂. Eggs were then transferred to 25 μl drops of G1 (Vitrolife) medium covered with paraffin oil (Irvine Scientific). The embryo culture was conducted up to the blastocyst stage in sequential G1 and G2 (Vitrolife) medium preequilibrated at 37°C and 6% CO₂. Embryos were kept in the G2 medium for 48 h before the transfer to pseudopregnant females.

The potential protective role of melatonin against ART-induced vascular dysfunction, melatonin (10⁻⁶ M; Sigma-Aldrich, St. Louis, MO) was added to the G1 and G2 culture media. The dose of melatonin used was based on previous studies in mice showing that addition of 10⁻⁶ M of melatonin to culture media had better effects on embryo culture development compared with higher or lower doses of melatonin (9).

Embryo transfer. NMRI females of at least 6 wk of age were placed with vasectomized males to mate 2.5 days before embryo transfer. The morning after mating, females were checked for the presence of a vaginal plug. On the transfer day, pseudopregnant females were anesthetized by IP injection of xylazine (15 mg/kg) and ketamine (100 mg/kg). Fourteen to twenty embryos were transferred to the uteri of each female.

Mesenteric artery function in vitro. Mesenteric artery function in vitro was assessed as previously described (20). Briefly, 12- to 14-wk-old male offspring of ART and control mice were euthanized with an overdose of pentobarbital sodium (200 mg/kg IP). The mesenteric artery was dissected free of parenchyma and cut into a ring. Each ring was positioned between two stainless steel wires (diameter 25 μm) in 5-ml organ bath of a small vessel myograph (DMT 620M, Danish Myo Technology, Aarhus, Denmark) (16). The organ bath was filled with modified Krebs-Ringer bicarbonate solution [composition (in mM): 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 11.1 glucose], maintained at 37 ± 0.5°C, and aerated with 95% O₂ plus 5% CO₂ (pH 7.4). At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension, corresponding to a transmural pressure of 100 mmHg. Arterial blood pressure was recorded during the transmural pressure measurement to the whole image. Nuclear mean intensities were measured by manually outlining all nuclei. Mean nuclear methylation intensity was expressed as the 5-methylcytosine-to-SYTOX Green signal ratio.

Methylation of the eNOS gene promoter in the femoral artery. Methylation of the eNOS gene promoter was done as described before (20). Briefly, 12- to 14-wk-old male offspring of ART and control mice were euthanized with an overdose of pentobarbital sodium (200 mg/kg IP). The mesenteric artery was dissected free of parenchyma and cut into a ring. Each ring was positioned between two stainless steel wires (diameter 25 μm) in 5-ml organ bath of a small vessel myograph (DMT 620M, Danish Myo Technology, Aarhus, Denmark) (16). The organ bath was filled with modified Krebs-Ringer bicarbonate solution [composition (in mM): 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 11.1 glucose], maintained at 37 ± 0.5°C, and aerated with 95% O₂ plus 5% CO₂ (pH 7.4). At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension, corresponding to a transmural pressure of 100 mmHg, and allowed to equilibrate for 1 h. The PowerLab/Labchart software (PowerLab/Labchart 30 Series, Danish Myo Technology) was used for data acquisition and display. Then acetylcholine- (ACH, 10⁻⁹ to 10⁻⁵ M) or sodium nitroprusside-induced (10⁻⁴ to 10⁻³ M) vasodilation was assessed in mesenteric arteries preconstricted with phenylephrine (10⁻⁶ mol/l) at a level corresponding at least to the maximal response to potassium (100 mmol/l KCl). The ACh- or sodium nitroprusside-induced change in tension was expressed as the percentage of the initial contraction induced by phenylephrine. To further examine the role of the nitric oxide (NO) pathway, we assessed the mesenteric artery vasodilator response in the presence of the NO synthase (NOS) inhibitor N⁴-nitro-L-arginine (L-NNA; Sigma-Aldrich, St. Louis, MO) (18). L-NNA (10⁻⁴ M) was added to the organ bath after equilibration.

Arterial blood pressure. Arterial blood pressure was recorded continuously in awake mice, as previously described (20). These measurements have been shown to closely reflect blood pressure measurements obtained by telemetry (20). Briefly, a fluid-filled PE-10 tubing connected to a pressure transducer was inserted into the carotid artery under isoprostane anesthesia and tunneled subcutaneously to exit at the back of the neck. Mice were allowed to recover for 4-5 h before the blood pressure measurement.

DNA methylation in four-cell embryos. Two days after IVF, embryos were processed for 5-methylcytosine immunodetection. To obtain in vivo embryos, 2 days after fertilization (presence of a vaginal plug), superovulated female mice were killed by cervical dislocation, the uteri were excised and flushed with HTF/HAS, and embryos were harvested. Embryos were washed in PBS, fixed for 15 min in fresh 4% paraformaldehyde (Sigma), and permeated with PBS-0.2% Triton X-100 (Sigma) for 30 min at room temperature. Subsequently, embryos were depurinated with 4 N HCl in PBS supplemented with 0.1% Triton X-100 solution at room temperature for 10 min, washed several times with PBS-0.05% Tween 20 (Sigma) (PBST), and placed overnight in a blocking solution containing PBS-2% BSA (Sigma) at 4°C. Methylated DNA was visualized with a mouse monoclonal antibody against 5-methylcytosine (Calbiochem NAA81). Embryos were incubated with this antibody at 37°C for 1 h (1:100 dilution in PBS-2% BSA), washed with PBS-2% BSA for at least 30 min, and incubated for 1 h at room temperature with an anti-mouse Alexa Fluor 647 dye (Invitrogen, Basel, Switzerland). After several washes in PBST, samples were submitted to ribonuclease A treatment (1 mg/ml at 37°C for 1 h) and washed again with PBST. Embryos were then mounted in Mowiol (Calbiochem, cat. no. 475904), supplemented with 2.5% 1,4-diazabicyclo-[2,2,2]-octane (Sigma) containing 0.1 mM SYTOX Green nucleic acid dye (Invitrogen).

Images were made with a LSM710 Zeiss microscope equipped with a ConfoCor 3 laser scanning microscope coupled to Zen2009 software (Carl Zeiss MicroImaging), using a Plan-Apochromat 100×/1.40 oil immersion objective (numerical aperture = 1.40) and excitation wavelengths of 488 and 633 nm. For each wavelength, serial optical sections (Z-series) were collected at 0.8-μm intervals through the embryos. Collection of each color channel was done sequentially, and the same gain, black-level, and aperture parameters were used for each experiment.

For quantitative measurements, the Z-series were merged with the IMARIS suite 6.3.1 software (Bitplane, St. Paul, MN) to produce a three-dimensional image depicting the staining patterns and total intensities of the nuclei. Using this software, the integrated fluorescence emitted by each nucleus was measured and corrected for background by subtracting the mean intensity of the cytoplasmic area to the whole image. Mean nuclear intensities were measured by manually outlining all nuclei. Mean nuclear methylation intensity was expressed as the 5-methylcytosine-to-SYTOX Green signal ratio.

Methylation of the eNOS gene promoter in the femoral artery. Methylation of the eNOS gene promoter was done as described before (20). Briefly, methylation analysis was performed in the eNOS (GenBank no. AF091262) core promoter, where DNA methylation was shown to control gene expression (3, 8). The following oligonucleotides were designed with the PyroMark Assay Design 2.0 program (Qiagen): eNOS-F, 5'-TGGAGTTTTGTTTGTATGGAT-GAT-3'; and eNOS-R, 5'-biotin-7'-CACAAATCCTAAC-CCTTTCCTTAA-3'. The biotinylated PCR products were purified using streptavidin-Sepharose beads (Amersham) and sequenced using the PSQ 96 Gold reagent kit (Biotage, Uppsala, Sweden) with the following oligonucleotide: eNOS-S, 5'-TTTGGTTTATTTATGGATGATT-3'. This sequence was analyzed by pyrosequencing was 151 bp long, encompassing 8 CpGs.

Protein extraction and eNOS Western blot. Protein extraction and eNOS Western blot were done as described before (20). Four carotid arteries of the same condition were pooled and grounded in liquid nitrogen. Each pool of carotid tissue was suspended in SDS lysis buffer (62.5 mM Tris pH 6.8, 5% SDS, 10 mM EDTA). Following sonication, and 5-min full-speed centrifugation, supernatant proteins were quantified by the Lowry method. Samples were heated at 95°C.
for 3 min and loaded in duplicate (8 μg/well) in a 8% acrylamide gel and transferred to PVDF membrane (Millipore). The membrane was blocked with 5% milk-Tris-buffered saline, 0.1% Tween (TBST) for 1 h. Blots were probed with anti-eNOS (1:1,000, BD Transduction Laboratories) in 5% milk/TBST overnight and were detected using peroxidase-conjugated secondary antibody (1:10,000) in 5% milk/TBST for 1 h. Products were visualized by chemiluminescence (GE Healthcare), and band intensity was measured using ImageQuant 5.0 software (Molecular Dynamics). Equal protein loading was confirmed by α-tubulin (1:10,000, Thermo Scientific Pierce Products) hybridization on the same membrane.

NOx plasma concentration. NOx was measured in plasma samples obtained by cardiac punctation by chemiluminescence with a NO analyzer (Sievers 280 NOA) after reduction of NOx to NO with VCl3 (20).

Statistical analysis. Statistical analysis was done with the Graphpad Prism 5 software package (GraphPad Software, San Diego, CA). Bivariate analyses were made using the unpaired two-tailed Student t-test. Multivariate analysis was made using one-factor or two-factor ANOVA. Post hoc comparisons were made using the Bonferroni t-test. Multivariate analysis was made using one-factor or two-factor ANOVA. Post hoc comparisons were made using the Bonferroni t-test.

RESULTS

Addition of melatonin to the culture media almost doubled the success rate of IVF (from 31 to 56%, ART vs. ART + melatonin, P = 0.02), whereas the number of pups remained unchanged (4.1 ± 2.4 vs. 4.9 ± 2.8 pups/mother, ART vs. ART + melatonin, P = 0.33) and was significantly lower than in mice having conceived naturally (8.1 ± 1.8 pups/mother, P ≤ 0.003 vs. ART or ART + melatonin).

Vascular function and arterial blood pressure. Figure 1 shows that, as expected (20), ACh-induced mesenteric artery vasodilation in vitro was impaired (Fig. 1A) in ART compared with control mice. Most importantly, addition of melatonin to the culture media prevented the impairment of ACh-induced vasodilation in ART mice. In the presence of L-NNA, endothelium-dependent vasodilation was abolished in the three groups (Fig. 1B). Endothelium-independent, sodium nitroprusside-induced mesenteric artery vasodilation was comparable in ART and control mice and remained unchanged after addition of melatonin to the culture media (Fig. 1C). Finally, in accordance with previously reported data (20), arterial blood pressure was increased in ART compared with control mice (109.5 ± 3.8 vs. 104.0 ± 4.7 mmHg, P < 0.01, ART vs. control). Addition of melatonin to the culture media prevented the increase of arterial blood pressure in ART mice (109.5 ± 3.8 vs. 104.6 ± 3.4 mmHg, P < 0.01, ART vs. ART + melatonin).

DNA methylation in four-cell embryos. There is evidence that culture-induced alterations of DNA methylation are already detectable during the very early development of the embryo (29). We, therefore, compared the methylation of embryos obtained 2 days after in vivo fertilization or IVF. Figure 2 shows that DNA methylation in ART embryos was significantly different from that in in vivo embryos. Addition of melatonin to the culture media prevented these alterations of the methylation in in vitro embryos.

Methylation of the eNOS gene, eNOS expression, and NOx plasma concentration. To examine whether the favorable cardiovascular effects of melatonin were related to prevention of epigenetic alterations of the eNOS gene, we compared DNA methylation of the promoter of the eNOS gene and eNOS expression in ART mice generated with/without the addition of melatonin to the culture media and in control mice. As expected (20), DNA methylation of the promoter of the eNOS gene (Fig. 3A) and eNOS expression (Fig. 3B) were altered in arterial tissue of ART mice and associated with decreased NOx plasma concentration in ART compared with control mice (Fig. 3C). Figure 3 shows that addition of melatonin to the culture media prevented the dysmethylolation of the promoter of the eNOS gene and decreased eNOS expression in arterial tissue and the alteration of the NOx plasma concentration.

DISCUSSION

ART induces vascular dysfunction in young apparently healthy children, which may predispose them to premature cardiovascular disease (20, 23, 25, 26). In industrialized countries, these children make up 2–5% of births, and there is an urgent need to find ways to prevent this problem. In mice, ART-induced epigenetic alterations that have been attributed to suboptimal culture conditions (15) contribute to cardiovascular dysfunction (20). Melatonin has been shown to protect oocytes against environmental insults (11, 31). Here, we found that the addition of melatonin to the culture media prevented epigenetic alterations and decreased expression of the eNOS gene, endothelial dysfunction, and arterial hypertension in ART mice. These findings provide proof of principle that, in mice, ART-induced epigenetic and cardiovascular alterations can be prevented, and that modification of the culture media offers a possibility to attain this important aim.

Fig. 1. Effects of the addition of melatonin to the cleavage and blastocyst development media on endothelium-dependent (acetylcholine) and -independent (sodium nitroprusside) mesenteric artery vasodilation in assisted reproductive technologies (ART) mice and control mice. Mesenteric artery responses to cumulative doses of acetylcholine (10−5−10−8 M) in the absence (A) or presence of a nitric oxide synthase (NOS) inhibitor (Nω-nitro-L-arginine: 10−4 M; B), and responses to the nitric oxide donor sodium nitroprusside (10−5−10−8 M; C) are shown in control and ART mice generated without or with addition of melatonin to the culture media. Values are means ± SE for at least 8 mice per group.

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00621.2014 • www.ajpheart.org
ART implies exposure of the embryo to environmental stress related to suboptimal culture conditions that are reflected by reduced pregnancy rates compared with in vivo embryos (15). In humans, oral administration of melatonin increases the fertilization rate in sterile women, an effect that has been attributed to protective effects on oocytes of this pineal gland product against environmental stress (32). The present observation in mice that adjunction of melatonin to the cleavage and blastocyst development media significantly increased the success rate of ART in mice (from 31 to 56%) is consistent with these observations in humans.

It has been shown that the prevalence of methylation disturbances in two-cell stage ART embryos is increased compared with that in embryos from normal mating (29), and their ability to maintain genetic imprinting is impaired compared with that of in vivo embryos (15), alterations that may contribute to cardiovascular dysfunction in ART mice (20). The present finding of altered methylation in ART embryos compared with in vivo embryos is consistent with these earlier observations. There is evidence that melatonin is involved in the epigenetic regulation of gene expression (13, 17, 28). In line with this concept, here, we found that addition of melatonin to the culture media prevented the altered methylation in ART embryos that was now comparable to the one observed in in vivo embryos. Collectively, these findings are consistent with the hypothesis that culture media are a primary culprit causing epigenetic alterations in ART embryos, and that modification of the culture media allows the prevention/attenuation of these deleterious effects of the culture media and/or some other possible culprit (temperature, altered O2/CO2 concentration, etc.) acting on the embryo during this critical period.

In line with previous findings, ACh-induced vasodilation was impaired, and arterial blood pressure was increased in ART mice compared with control animals (20). This vascular dysfunction in ART mice was endothelium dependent because endothelium-independent vasodilation as assessed by a NO donor (sodium nitroprusside) was comparable between the three groups. Moreover, in the presence of NOS inhibitor (l-NNA), endothelium-dependent vasodilation was abolished, further highlighting the role of NO-mediated relaxation in this setting. In previous studies in mice, mesenteric artery vasodilation in the presence of a NOS inhibitor, while significantly diminished, remained detectable, and, depending on the study protocol, the residual vasodilation reported was between 10 and 60% (4, 21, 35, 36). In the present study, the residual maximal ACh-induced vasodilation in the presence of l-NNA was smaller than in all previous reports of which we are aware, namely 9.6 ± 13.8%. Of note, however, these previous studies were performed in C57/Bl6 mice, whereas the present study was performed in FVB mice, suggesting that this difference could be related to different mesenteric artery responsiveness to NOS inhibition between mouse strains. In line with this speculation, we found that, in C57/Bl6 mice, NOS inhibition, while causing a significant shift of ACh-induced mesenteric artery vasodilation, was much smaller than in previous reports (4, 21, 35, 36). This finding is consistent with the hypothesis that FVB mice have a lower basal vasoactivity, which could be related to differences in the expression of NO synthase isoforms between mouse strains.

ART implies exposure of the embryo to environmental stress related to suboptimal culture conditions that are reflected by reduced pregnancy rates compared with in vivo embryos (15). In humans, oral administration of melatonin increases the fertilization rate in sterile women, an effect that has been attributed to protective effects on oocytes of this pineal gland product against environmental stress (32). The present observation in mice that adjunction of melatonin to the cleavage and blastocyst development media significantly increased the success rate of ART in mice (from 31 to 56%) is consistent with these observations in humans.

It has been shown that the prevalence of methylation disturbances in two-cell stage ART embryos is increased compared with that in embryos from normal mating (29), and their ability to maintain genetic imprinting is impaired compared with that of in vivo embryos (15), alterations that may contribute to cardiovascular dysfunction in ART mice (20). The present finding of altered methylation in ART embryos compared with in vivo embryos is consistent with these earlier observations. There is evidence that melatonin is involved in the epigenetic regulation of gene expression (13, 17, 28). In line with this concept, here, we found that addition of melatonin to the culture media prevented the altered methylation in ART embryos that was now comparable to the one observed in in vivo embryos. Collectively, these findings are consistent with the hypothesis that culture media are a primary culprit causing epigenetic alterations in ART embryos, and that modification of the culture media allows the prevention/attenuation of these deleterious effects of the culture media and/or some other possible culprit (temperature, altered O2/CO2 concentration, etc.) acting on the embryo during this critical period.

In line with previous findings, ACh-induced vasodilation was impaired, and arterial blood pressure was increased in ART mice compared with control animals (20). This vascular dysfunction in ART mice was endothelium dependent because endothelium-independent vasodilation as assessed by a NO donor (sodium nitroprusside) was comparable between the three groups. Moreover, in the presence of NOS inhibitor (l-NNA), endothelium-dependent vasodilation was abolished, further highlighting the role of NO-mediated relaxation in this setting. In previous studies in mice, mesenteric artery vasodilation in the presence of a NOS inhibitor, while significantly diminished, remained detectable, and, depending on the study protocol, the residual vasodilation reported was between 10 and 60% (4, 21, 35, 36). In the present study, the residual maximal ACh-induced vasodilation in the presence of l-NNA was smaller than in all previous reports of which we are aware, namely 9.6 ± 13.8%. Of note, however, these previous studies were performed in C57/Bl6 mice, whereas the present study was performed in FVB mice, suggesting that this difference could be related to different mesenteric artery responsiveness to NOS inhibition between mouse strains. In line with this speculation, we found that, in C57/Bl6 mice, NOS inhibition, while causing a significant shift of ACh-induced mesenteric artery vasodilation, was much smaller than in previous reports (4, 21, 35, 36). This finding is consistent with the hypothesis that FVB mice have a lower basal vasoactivity, which could be related to differences in the expression of NO synthase isoforms between mouse strains.

Fig. 2. A: DNA methylation in four-cell mouse embryos obtained by in vitro fertilization, with and without addition of melatonin to the culture media, and in control embryos obtained by in vivo fertilization. Methylated DNA was visualized with an antibody against 5-methylcytosine (5 MeC). B: quantification of total nuclear methylation intensity in four-cell mouse embryos obtained by in vitro fertilization with and without addition of melatonin to the culture media, and in control embryos obtained by in vivo fertilization. Values are means ± SE. NS, nonsignificant.

Fig. 3. Effects of the addition of melatonin to the cleavage and blastocyst development media on endothelial NOS (eNOS) promoter gene methylation, eNOS expression, and nitrate/nitrite (NOx) plasma concentration in ART mice. Methylation of the promoter of the eNOS gene in the femoral artery (A), Western blot analysis of vascular eNOS protein expression (B), and NOx plasma concentration (C) are shown in control mice and ART mice generated without or with addition of melatonin to the culture media. Values are means ± SE for at least 8 mice per group.
artery dilation (from roughly 94% to 33%), did not abolish the relaxation. These data demonstrate significantly (ANOVA, P = 0.039) different effects of NOS inhibition on mesenteric artery vasodilation between FVB and C57Bl6 mice that need to be taken into account in future studies. These cardiovascular alterations in ART mice have been shown to be related to epigenetic alterations of the eNOS gene, resulting in decreased vascular eNOS expression and NOx synthesis, because restoration of normal eNOS gene methylation by administration of a histone deacetylase inhibitor normalized cardiovascular function in ART mice (20). Here, we found that addition of melatonin to the culture media prevented epigenetic alterations of the eNOS gene, and, in turn, impaired vascular eNOS expression and NOx synthesis, endothelial dysfunction, and arterial hypertension in ART mice. These findings provide further evidence for the important role played by epigenetic alteration of the eNOS gene in causing ART-induced cardiovascular dysfunction. In line with this concept, there is indeed abundant data demonstrating that altered plasma NO bioavailability is associated with altered cardiovascular homeostasis and insulin resistance in experimental animal models and humans (5, 7, 27). Finally, we cannot exclude the possibility that ART may alter the function of other genes involved in cardiovascular regulation. However, since, in our laboratory’s previous studies, (20) we did not detect any alteration of the methylation of the endothelin-1 and the angiotensin converting enzyme gene, we did not assess potential effects of melatonin on the methylation of these genes in the present study.

Conclusion. ART-induced cardiovascular dysfunction represents a major problem with possibly dramatic consequences on future cardiovascular morbidity and mortality in this rapidly growing population. Here, we found that, in mice, ART-induced vascular dysfunction and arterial hypertension can be prevented by modification of the culture media. This favorable effect appears to be related, at least in part, to prevention of ART-induced epigenetic alterations of the regulation of the eNOS gene. There is evidence that ART-induced vascular dysfunction in humans may be related to a similar mechanism (22). We speculate that the modification of the culture media may allow the prevention of ART-induced premature atherosclerosis in humans. To attain this aim, there is an urgent need to obligate regulation of culture media to declare their exact composition and fertilization clinics to keep mandatory and detailed registers of the culture conditions used.

ACKNOWLEDGEMENTS
We are indebted to Dr. Françoise Urner for help with the initial studies, and to Caroline Mathieu and Christelle Stouder for invaluable technical assistance.

GRANTS
This work was supported by the Swiss National Science Foundation, the Placide Nicod Foundation, the Eagle Foundation, the Leenaards Foundation, the Swiss Society of Hypertension, and the Swiss Society of Cardiology.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


