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Participation of the Mouse Implanting Trophoblast in Nitric Oxide Production During Pregnancy[1]

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ABSTRACT

While considerable progress has been made in elucidating nitric oxide (NO) regulatory mechanisms in the later stages of gestation, much less is known about its synthesis and role during embryo implantation. Thus, to evaluate the participation of the trophoblast in the production of NO during this phase, this study focused on NADPH-diaphorase activity and the distribution of NO synthase isoforms (NOS) using immunohistochemistry in pre- and postimplantation mouse embryos in situ and in vitro, as well as on NO production itself, measured as total nitrite, in trophoblast culture supernatants (Griess reaction). No NADPH-diaphorase activity was found in preimplanting embryos except after culturing for at least 48 h, when a few trophoblastic giant cells were positive. Conversely, postimplantation trophoblast cells either lodged into the implantation chamber (in situ) or after culturing (in vitro) showed intense NADPH-diaphorase activity. Also in the postimplantation trophoblast, the endothelial and inducible NOS (eNOS and iNOS) isoforms were immunodetected, under both in situ and in vitro conditions, although in different patterns. Extracts of ectoplacental cone also revealed bands of 135 and 130 kDa on SDS-PAGE that reacted with anti-eNOS and anti-iNOS, respectively, on Western blot. Analysis of the culture supernatant demonstrated that the nitrite concentration was 1) proportional to the number of cultured trophoblast cells, 2) almost completely abolished in the presence of N^{ω}-nitro-L-arginine methyl ester, and 3) increased 2-fold in cultures stimulated with γ-interferon. These results strongly suggest the production of NO from constitutive and inducible isoforms of NOS by the implanting mouse trophoblast. They also emphasize the possibility of the participation of these cells in vasodilatation and angiogenesis, and in cytotoxic mechanisms involved in the intense phagocytosis of injured maternal cells, which occur during the implantation process.

INTRODUCTION

In a large variety of tissues, an important role has been suggested for nitric oxide (NO) in many physiological functions including vasodilatation, relaxation of nonvascular smooth muscle, inhibition of platelet aggregation, inhibition of leukocyte adhesion and rolling, neurotransmission, and mediation of cytotoxic macrophage effects [1–3]. Coincidentally some of these events occur during pregnancy, and the participation of NO in mammalian gestation has been demonstrated in several studies [4–9].

In the placenta, the maintenance of vascular tone, the attenuation of vasoconstriction, and the prevention of platelet and leukocyte adhesion to the trophoblast surface seem to be the main functions of NO generated at the fetal-maternal interface [10–13]. Similarly, the possible participation of NO in uterine blood flow also may be relevant during implantation. The action of NO as a cytotoxic molecule [1–3] in the phagocytosis exhibited by the implanting, hemochorial trophoblast should also be considered. In mice, phagocytic activity by the trophoblast, temporally limited and very intensely expressed during early pregnancy, apparently facilitates the invasion steps, provides a nutritional source for embryo development before completion of the placenta, and also participates in embryo protection [14–16]. Reactive oxygen species (ROS) recently have been suggested as mediators in this process [17,18], much as occurs in polymorphonuclear leukocytes and macrophages [19–24]. Under stimulation, trophoblast cells from implanting embryos produce and release significant amounts of cytotoxic ROS such as hydrogen peroxide and the superoxide anion [17,18]. Although NO is a member of the ROS family of molecules and exhibits a potent, cytotoxic effect on target cells to further facilitate the phagocytic process [24], its potential role has not been evaluated. However, the presence of this cytotoxic molecule in the trophoblast invasion pathway might influence the fate of surrounding maternal cells, allowing or even triggering their subsequent internalization by the trophoblast.

Furthermore, a remarkable vascular rearrangement, neovascularization, and vasodilatation take place in the endometrium surrounding the embryo [25] concomitant with the implantation process. In addition, during this period in many species, the outermost embryonic layer formed by trophoblast giant cells (TGCs) invades the endometrium reaching and breaching the uterine vessels, maintains a close relationship with the maternal endothelium, and frequently lines the endometrial vessels [25–30]. This strategic position of the trophoblast interacting with vascular components and in direct contact with the maternal blood makes this cell population an attractive target for studies of the mechanisms that participate in the regulation of circulatory dynamics during the maternal-embryonic interrelationship.

Regarding endometrial vasodilatation, various components of maternal origin have been considered as playing a role of fundamental importance to the development of the embryo and placenta [31,32]. However, the participation of the embryonic organism should not be underestimated.

These considerations led us to examine whether the rodent trophoblast produces and releases NO, which might participate in the dynamics of maternal circulation, acting to maintain adequate blood flow during early pregnancy and mediating the phagocytic activity of these cells.

To test this hypothesis, in the present work we detected the presence of possible sites of NO synthase (NOS) activ-

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ity (NADPH-diaphorase enzyme complex), using a histochemical technique, and investigated the endothelial and macrophage isoforms of NOS in pre- and postimplantation mouse embryos using immunohistochemistry. The release of NO was also evaluated in supernatants of cultured trophoblast.

MATERIALS AND METHODS

Antibodies and Solutions

Fetal calf serum (FCS) was purchased from Cultilab Inc. (Campinas, SP, Brazil). Tris, sodium nitrite, phosphoric acid, sulfanilamide, and *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) were obtained from E. Merck (Darmstadt, Germany). MEM nonessential amino acids, insulin, uridine, penicillin, and streptomycin were purchased from Gibco BRL (Grand Island, NY). Glycol methacrylate resin was obtained from LKB (Bromma, Sweden) and hCG from Organon (São Paulo, SP, Brazil). Polyclonal endothelial NOS (eNOS) antibody and polyclonal macrophage NOS (iNOS) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated goat anti-rabbit IgG, streptoavidin-alkaline phosphatase complex kit, Fast Red TR/Naphtol AS-MX developer kit, β-NADPH, N^{ω}-nitro-L-arginine methyl ester (L-NAME), PBS, BSA, Dulbecco's modified Eagle's medium (D-MEM), L(+) lactic acid, pyruvic acid, adenosine, guanosine, thymidine, recombinant mouse gamma-interferon (γ-IFN), nitro blue tetrazolium (NBT), paraformaldehyde, poly-L-lysine solution, paraplast, and gonadotropin from eCG were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals

Virgin female Swiss mice (F1 NKW × AKR) aged 3–4 mo were induced to superovulate by i.p. injections of 5 IU of eCG and 7.5 IU of hCG administered 48 h apart. These females were caged overnight with males (F1 Balb/c × AJ), and successful mating was verified on the following morning. The presence of a vaginal plug established the first half-day of pregnancy (Day 0.5). Food and water were available ad libitum. The photoperiod was 12L:12D. A total of 177 pregnant females were used to perform this experimental study.

Pregnant females were killed by cervical dislocation, and their uteri were immediately dissected in PBS containing 0.3% BSA. All procedures were performed in accordance with the ethical principles for animal research adopted by the Brazilian College of Animal Experimentation.

Preimplantation Embryo Collection and Culture

On Day 3.5 of pregnancy, preimplantation embryos were flushed from the uterine horns with PBS-10% FCS. Late morulas and early blastocysts (n = 186) were selected by stereomicroscope, and portions of them were cultured in lots of 4, in a 12-well multidish culture plate containing removable 18-mm-diameter glass coverslips. The embryos were cultured at 36.5°C in a humid atmosphere of 5% CO_2 in air in D-MEM medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% MEM nonessential amino acids, 0.4 μg/ml insulin, 1% nucleosides (adenosine, thymidine, guanosine, uridine), 520 μg/ml L(+) lactic acid, 56 μg/ml pyruvic acid, and 4 mg/ml BSA.

Freshly collected and 48- and 72-h-cultured embryos were used for NO and NOS evaluation. The experiments were repeated at least three times, on different occasions.

Ectoplacental Cone Collection and Culture

Embryos (n = 384) were surgically dissected under a stereomicroscope from the uteri of females killed by cervical dislocation on Day 7.5 of pregnancy. The ectoplacental cones (ECs) were then carefully separated from the surrounding embryonic tissues and cultured as previously described for blastocysts. Three to five ECs were introduced into each well of a 12-well multidish culture plate covered with removable 18-mm-diameter glass coverslips.

Implantation Sites on Day 7.5 of Pregnancy

The uteri of females on Day 7.5 of the pregnancy were collected immediately after death. The implantation chambers (n = 190) were removed and manually sliced into thin transverse fragments under Tris-buffered saline (TBS)-BSA. Some of the material was processed using immunohistochemical techniques, and the remainder was used for histochemical assays.

Histochemical Assay for NADPH-Diaphorase Activity

The histochemical assay for NADPH-diaphorase was performed in unfixed and fixed tissues at different stages of pregnancy. The fixed specimens were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature.

The enzyme complex activity was investigated in flushed blastocysts, in isolated embryos on Day 7.5 of pregnancy, in 24- and 96-h-cultured ECs, and in 24- to 72-h-cultured blastocysts.

After rinsing in PBS, the specimens were incubated for 30 min at room temperature in 1.0 mM β-NADPH and 0.25 mM NBT in PBS, pH 7.4 [12]. The specificity of the reaction was controlled by omitting the β-NADPH from the incubation solution. Reactions were also assayed in specimens previously incubated with L-NAME (10 mM, for 10 min at room temperature), the competitive inhibitor of both NOS isoforms [33].

After incubation, fragments of implantation sites and isolated embryos from Day 7.5 of pregnancy were prepared for embedding in glycol-methacrylate resin according to the manufacturer's instructions and examined as stained and unstained sections of 5-μm thickness.

The cultured ECs and blastocysts that had adhered to the glass coverslips were rinsed, counterstained with neutral red or left unstained, dried, and mounted in PBS/glycerol (1:9) and directly observed by conventional light microscopy.

Immunohistochemistry

Freshly collected and cultured embryos from Days 3.5 and 7.5 of pregnancy that were adhering to the glass coverslips were also assayed after rapid fixation (10 min) in a solution containing 60% methanol, 30% chloroform, and 10% acetic acid.

Implantation sites from Days 3.5 and 7.5 of pregnancy containing embryos were investigated in 5-μm-thick paraplast sections. The material was fixed by immersion in 4% paraformaldehyde in TBS, pH 7.4, for 24 h, followed by dehydration in ethanol and embedding.

The specimens were first incubated in either TBS-0.3% Triton plus 1% BSA or normal rabbit serum in TBS-Triton for 1 h, at room temperature, followed by incubation for 15 min in 15% acetic acid in water, to block endogenous

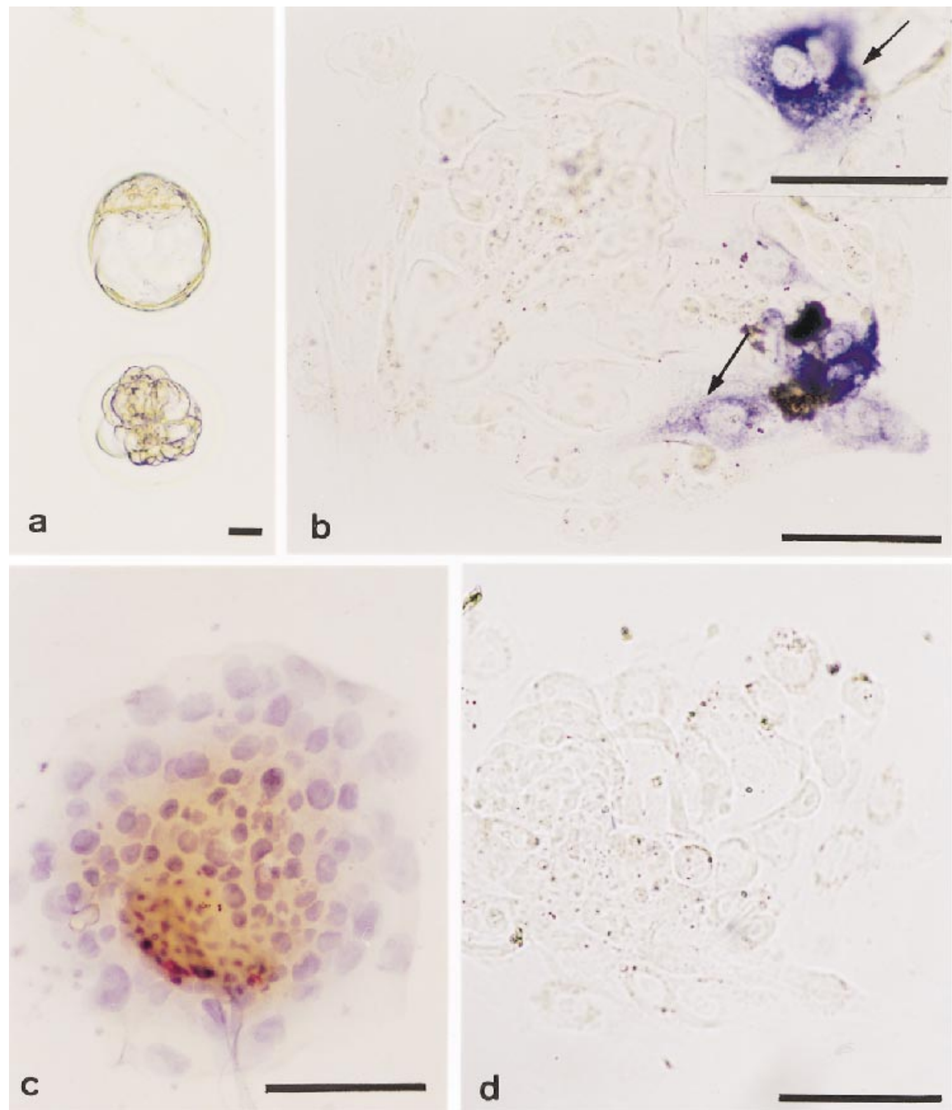

FIG. 1. NADPH-diaphorase histochemical staining showed no reactivity in the morulas and blastocysts collected on Day 3.5 of pregnancy (**a**). After 48 h of culture (**b**), only a few cells (arrows) were positive. Endothelial NOS (**c**) and iNOS (**d**) were not immunodetected in 48-h-cultured blastocysts. **c**) Mayer's hematoxylin counterstaining. Bars = 80 μm.

alkaline phosphatase activity. They were then incubated overnight at 4°C in polyclonal eNOS antibody (1:25) and in polyclonal iNOS antibody (1:50).

Antibody binding was detected using biotinylated goat anti-rabbit IgG (1:100) in TBS and streptoavidin-alkaline phosphatase complex (1:40). The presence of alkaline phosphatase was revealed using a Fast Red kit. Sections were counterstained with Mayer's hematoxylin for 5 min and then washed in distilled water. The slides and glass coverslips containing the material were then dried and mounted in PBS/glycerol (1:9) and observed in a light microscope.

Immunohistochemical controls consisted of substituting the primary antibody or the biotinylated anti-rabbit serum by TBS. Positive controls used histological preparations of lung tissue. Negative controls were performed using a nonrelevant antibody (polyclonal goat anti-rabbit IgG against *Toxoplasma gondii*).

Western Blotting

Western blot analysis was used to verify the presence of NOS isoforms in the ECs as well as the specificity of the rabbit antibodies to iNOS and eNOS [9]. Briefly, 300 ECs were solubilized, and the lysates were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Positive controls consisted of iNOS and eNOS antigens using the same procedure. The blots were incubated with either anti-iNOS immunoglobulin or anti-eNOS immunoglobulin (both at 20 μg/lane). Bound antibodies were detected using an alkaline phosphatase kit according to the manufacturer's instructions.

Evaluation of NO Production by Nitrite Measurement

The presence of nitrite, i.e., the end-product of the L-arginine-NO pathway, was estimated in the supernatants of cultured ECs. After culturing for 24 h to adhere and spread on the glass coverslip, ECs were exposed to 300 μl of either fresh medium or medium containing γ-IFN (100 U/ml) or L-NAME (1 mM) for an additional period varying from 24 to 72 h.

Lots of 1, 2, and 3–4 ECs in a total of 180 specimens were employed, and aliquots of the supernatant were collected after 24, 48, and 72 h of culture. The aliquots were filtered and stored at −20°C. All experiments were carried out in triplicate and were repeated at least three times on separate occasions.

Since NO is spontaneously oxidized to nitrite and nitrate, nitrite levels were measured as a reference value for NO released into the culture medium. Nitrite was measured us-

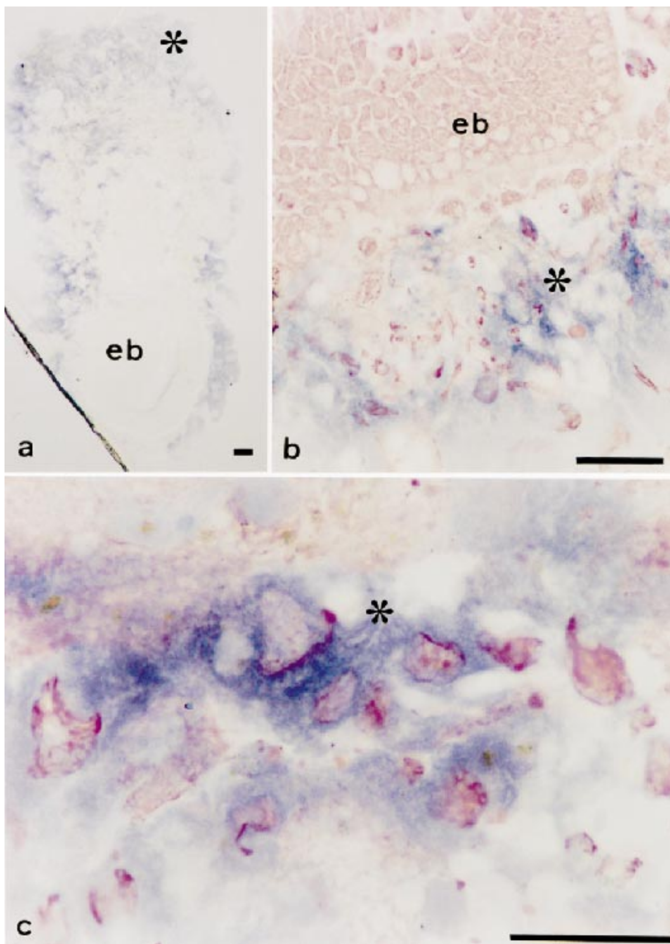

FIG. 2. NADPH-diaphorase in an isolated mouse embryo from Day 7.5 of pregnancy (**a–c**). Reactivity was observed only in the trophoblast (*) and mainly in the TGCs. No other embryonic cell population (eb) was positive. **a**) Unstained embryo. **b, c**) Neutral red counterstaining. Bars = 50 μm.

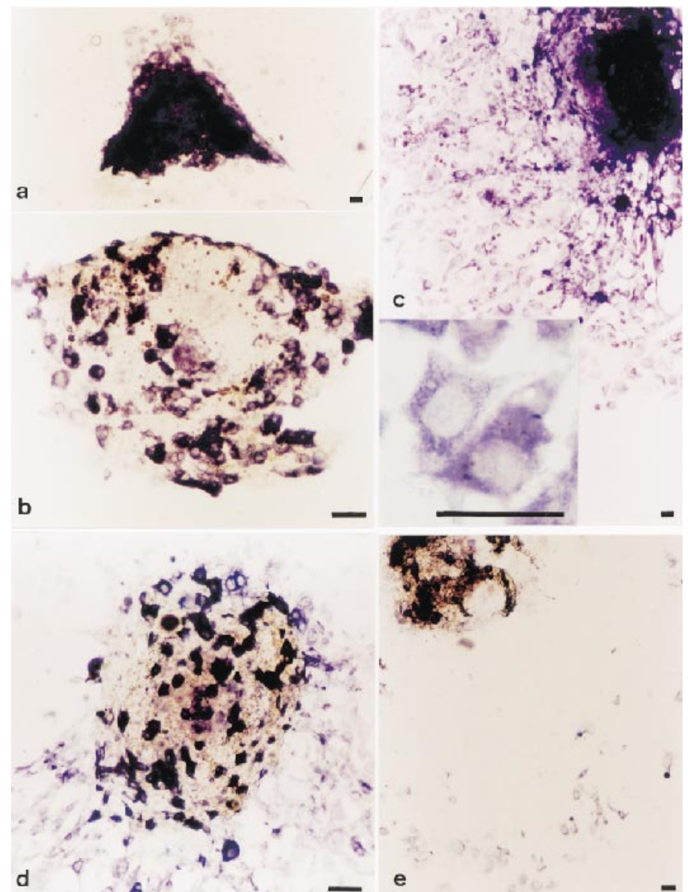

FIG. 3. Trophoblast cells from the EC (**a**) and mural region (**b**) are seen stained for diaphorase in an isolated embryo from Day 7.5 of pregnancy. In **c** and **d,** note the reactivity in the trophoblast cells after 48 h (**c**) and 72 h of culture (**d**). In the inset in **c,** the reaction can be clearly seen in the cytoplasm of these cells. The diaphorase reaction weakened after incubation with L-NAME (**e**). Bars = 50 μm.

ing the Griess reaction [34], which consisted of spectrophotometric measurement of absorbance at 570 nm of the purple azo dye formed after addition of the Griess Reagent (1% sulfanilamide in 5% phosphoric acid, 0.1% NED in water) to aliquots of the supernatant (1:1).

Sodium nitrite was prepared in concentrations of 5, 10, 20, and 40 μM in supplemented culture medium and spectrophotometrically measured to convert sample readings to μM nitrite.

Data from the experimental and control cultures were grouped and compared using the Tukey test after an ANOVA procedure was performed. Differences were considered significant at $P \leq 0.05$. Results are given as the mean ± SD.

RESULTS

NADPH-Diaphorase Activity

NADPH-diaphorase activity was demonstrated exclusively in the trophoblast cell population in the postimplantation period of pregnancy.

Blastocyst-stage embryos, enveloped by the zona pellucida, showed no NADPH-diaphorase activity (Fig. 1a); however, some enzymatic activity was occasionally present in the hatched 48-h-cultured embryos (Fig. 1b). When present, enzymatic reactivity was found exclusively in a few peripheral giant cells.

Postimplantation embryos from Day 7.5 of pregnancy showed strong NADPH-diaphorase activity. This reactivity was exclusively found in the trophoblast cells (Fig. 2a) located around the entire embryoblastic region (EC and mural region). The specific localization of the enzymatic activity in the trophoblast could be clearly seen in the methacrylate resin sections (Fig. 2, b and c). Among the trophoblast cell types present at this moment of pregnancy, the giant peripheral cells appear to be more intensely involved in NADPH-diaphorase staining (Fig. 2c).

The detection of the enzymatic complex in cultured ECs also showed strongly positive trophoblast cells in the first 48 h of in vitro conditions (Fig. 3, a–c). However, apparently, the number of diaphorase-positive cells decreased in the subsequent periods (Fig. 3d), reaching a minimum of a few sparse, positive cells after 96 h of culture (data not shown). Whether the NADPH-diaphorase staining was cytoplasmic or whether there were also sites of membrane reactivity could not be resolved by light microscopy (Fig. 3c, inset).

Previous treatment with L-NAME inhibited most NADPH-diaphorase activity in the EC cells, leaving only a few peripheral giant cells positive after 48 h of culture (Fig. 3e).

Fixed and unfixed specimens showed similar results. Re-

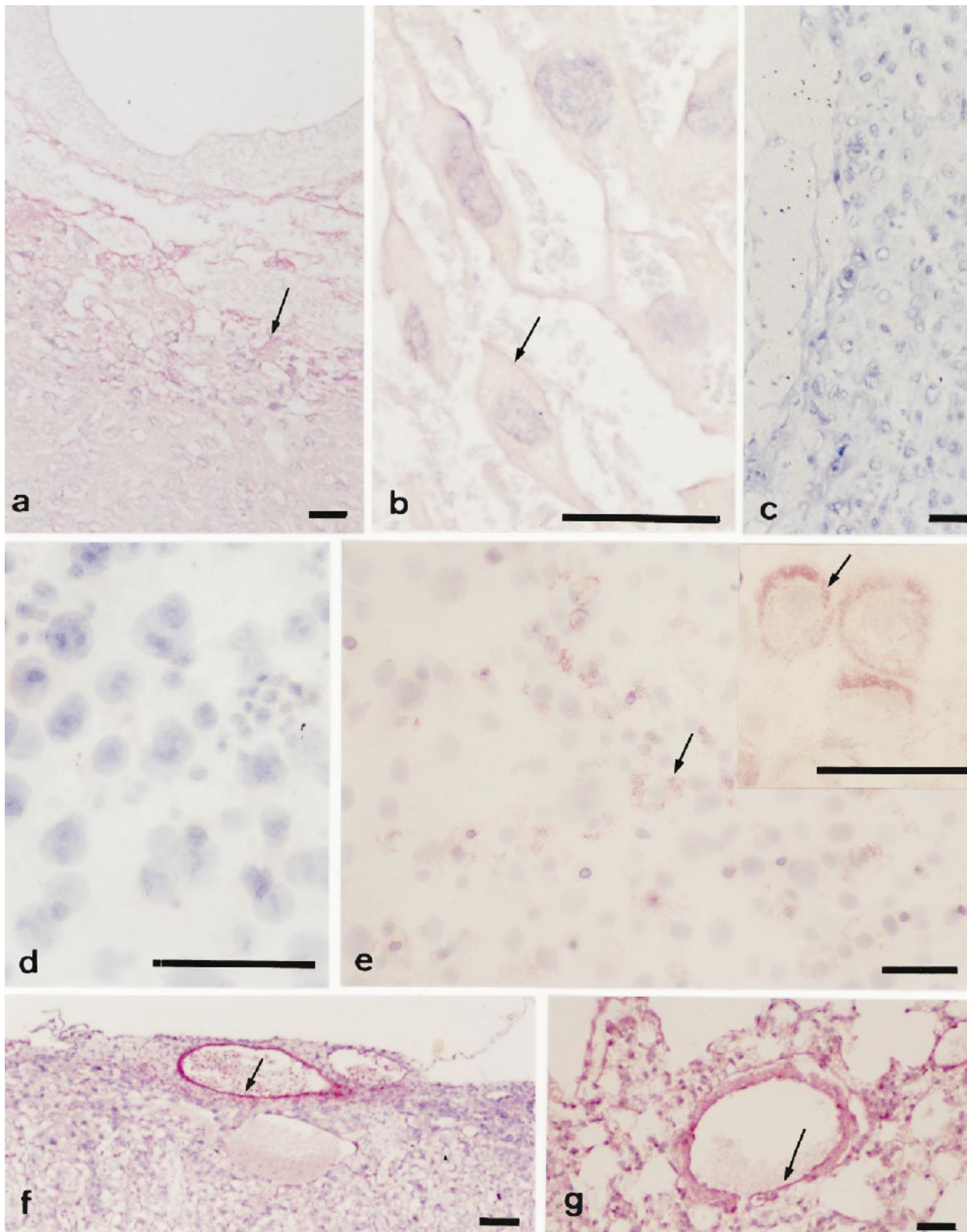

FIG. 4. Immunolocalization of eNOS in implantation sites on Day 7.5 of pregnancy (**a–c**) and in 48-h-cultured ECs (**d, e**). Labeling for eNOS was present in the TGCs (arrows) of both implantation sites (**a, b**) and in 48-h-cultured ECs (**e**). **c, d**) Negative controls. **f, g**) Positive controls; arrows indicate reactivity in the endothelial cells of the placenta (**f**) and lung vessels (**g**). Bars = 40 μm.

moval of β-NADPH from the incubation medium (control) resulted in a significant loss of NADPH-diaphorase staining.

Immunohistochemistry

No evidence for eNOS and iNOS isoforms was found in freshly collected 3.5-day-old blastocysts or in 24- to 72-h-cultured blastocysts (Fig. 1, c and d). In contrast, trophoblast cells from embryos on Day 7.5 of pregnancy were immunoreactive for both isoforms of NOS, under both in vitro and in situ conditions, predominantly in the TGCs (Fig. 4, a–e, and Fig. 5, a–d). The pattern of the immunostaining, however, seemed to exhibit slight differences depending on the NOS isoform investigated, the localization

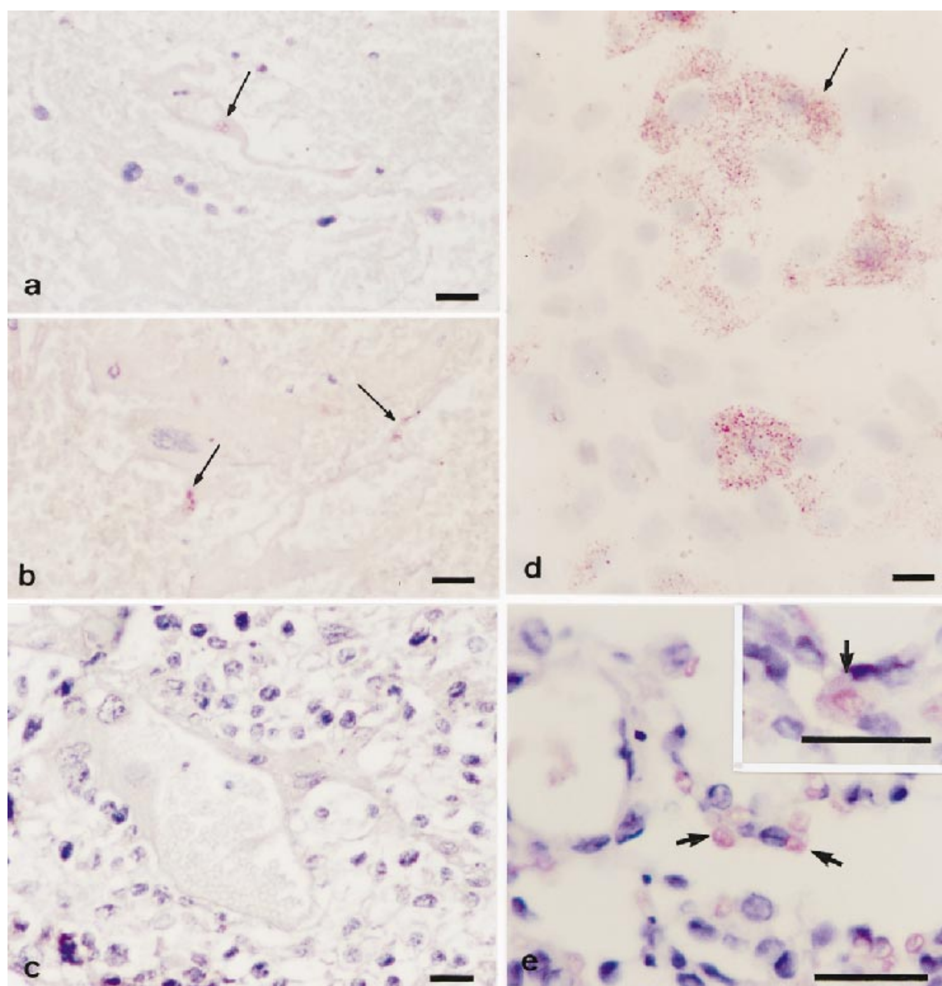

FIG. 5. Immunolocalization of iNOS in implantation sites on Day 7.5 of pregnancy (**a–c**) and in 48-h-cultured ECs (**d**). Immunolabeling was present as patches in the cytoplasm (**a, b, d**) of the TGCs (arrows). **c**) Negative control. **e**) Positive control; arrows indicate reactivity in macrophages of the lung. Bars = 25 μm.

of the labeled cells around the embryo, and the maintenance of the cells in culture.

TGCs from embryos on Day 7.5 of pregnancy, lodged at the implantation sites, exhibited strong labeling for eNOS in the mural region (Fig. 4a) and a weak, diffuse cellular reaction throughout the cytoplasm with a delicate but distinct positive zone surrounding the cell boundary of the polar cells (Fig. 4b). In contrast, the staining for iNOS was patchy in distribution independent of the localization of the TGCs around the embryo (Fig. 5, a and b).

Trophoblast cells from cultured ECs presented a granular cytoplasmic labeling for both eNOS and iNOS isoforms (Figs. 4e and 5d). The staining for eNOS, however, was apparently more concentrated in the peripheral areas of the cytoplasm (Fig. 4e, inset), while for iNOS the positive granular reaction was regularly distributed throughout the cytoplasm (Fig. 5d).

Endothelial activity appeared in several blood vessel types, either with or without a smooth-muscle sheath, in the maternal, placental, and lung tissues, confirming the reactivity of the primary anti-eNOS antibody (Fig. 4, f–g). Macrophages of the lung (Fig. 5e, inset) and liver exhibited strong positivity for iNOS. There was no evidence of reactivity in the negative controls in all specimens evaluated (Fig. 4, c and d, and Fig. 5c), or in those in which the nonrelevant antibody was used.

Nitrite Measurement

The ability of the trophoblast to produce NO was evaluated by estimating the amount of nitrite in the supernatant of trophoblast cells cultured under different experimental conditions. Nitrite production was directly proportional to the number of ECs cultured and was also a function of culture time (Fig. 6, a and b). The total amount of nitrite, however, was significantly reduced when the ECs were cultured in the presence of L-NAME; ECs challenged with γ-IFN showed a 2- to 4-fold increase in NO production (Fig. 6c).

Western Blotting

On Western blotting, bands of 130 and 135 kDa were identified in the EC tissue and recognized by iNOS and eNOS antibodies, respectively (Fig. 7).

DISCUSSION

In light of recent studies focused on NO production and emphasizing its crucial importance during the various stages of pregnancy [3–9,35–38], we investigated the participation of the mouse trophoblast in NO synthesis and pregnancy-associated functions. Our results clearly demonstrate that the trophoblast cells and, mainly, the TGCs of the postimplantation stage are able to contribute significantly to the source of gestational NO.

In this study, preimplanting, flushed blastocysts showed no diaphorase activity or immunostaining for endothelial and macrophage isoforms of NOS. NADPH-diaphorase was found exceptionally after 48 h of culture when only a few peripheral giant cells showed reactivity. Although NADPH-

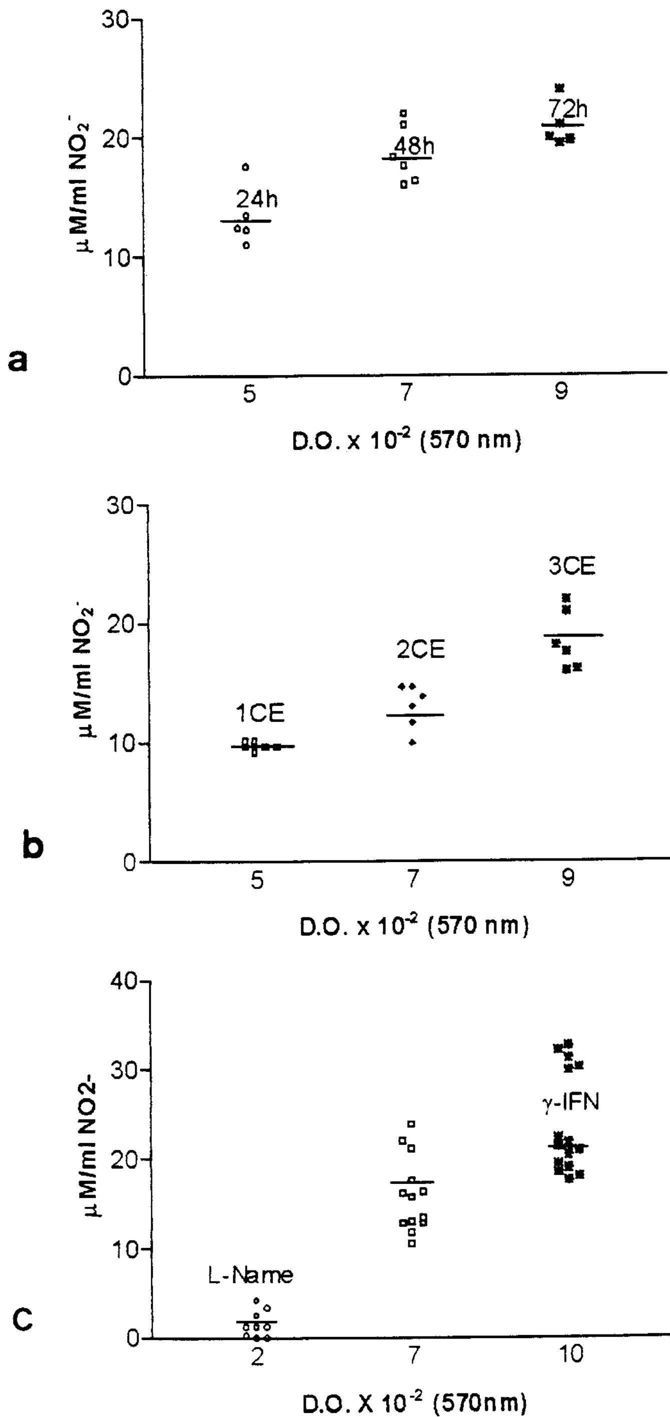

FIG. 6. Mean values (n = 3–5) of EC supernatant nitrite (NO^{-}_{2}) concentrations (μmol/ml) in the following situations: **a**) 3–4 ECs cultured for 24, 48, and 72 h; **b**) cultures of 48 h containing 1, 2, and 3 ECs; **c**) 3–4 ECs after 48 h of culture, incubated with L-NAME (circles, 0.1 M), with recombinant γ-IFN (asterisks, 100 U/ml), and with culture medium without inhibitors or stimulation (squares). The bars represent the median values for each experimental lot.

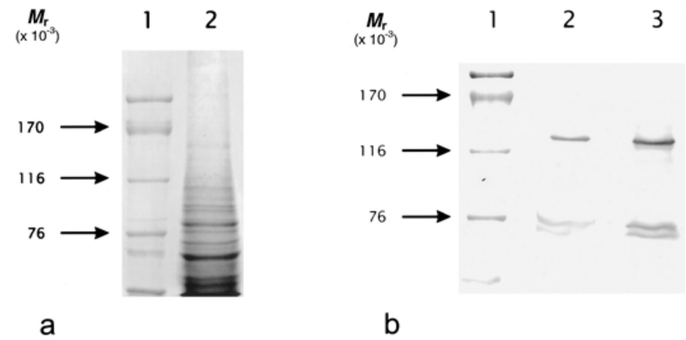

FIG. 7. SDS-PAGE (**a**) and Western immunoblotting (**b**) for eNOS and iNOS in ECs. A total of 300 solubilized ECs were submitted to electrophoresis, Western blotting, and immunostaining for eNOS (lane 2) and iNOS (lane 3) using polyclonal antibodies. Lane 1, molecular weight markers.

diaphorase is a component of the NOS complex and therefore should present colocalization with NOS protein [11,39–43], immunostaining for any NOS isoform was not detected in the cultured blastocysts.

To interpret this apparent inconsistency in our experimental data, at least two points may be considered: 1) at least in part, the expression of the diaphorase enzymatic complex might be associated with other dehydrogenases not specifically related to the NOS isoforms, such as flavin-containing oxidase, as suggested by Kristoffersen and Ulvestad [44]; 2) the diaphorase-reactive cells of cultured blastocyst might be expressing small amounts of NOS isoforms, more easily detectable by histochemistry than by immunohistochemistry procedures. Similar studies corroborate this possibility [44]. Assuming that the cultured blastocyst cells display a NADPH-diaphorase specific reactivity, the presence of a reaction product exclusively in occasional TGCs could result from the differentiation stage in culture conditions, or even from previous factors of signaling exerted by the maternal environment.

While the present study was being written, Gouge et al. [45] reported that trophoblast cells from Day 4-delayed embryos, recovered after 1-h exposure to estradiol-17β (E_2) treatment in utero, immunostained for endothelial and induced isoforms of NOS. The authors suggested that the synthesis of NOS is E_2-dependent, which could explain our results if we suppose that at the time of recovery the 3.5-day-old blastocysts had not yet been completely sensitized by maternal estrogens.

As previously shown by another study on a similar stage of pregnancy [46], mouse postimplanting embryos on Day 7.5 of pregnancy, conversely to preimplanting blastocysts, exhibit NADPH diaphorase activity. The reaction was restricted to the trophoblast population, being more evident and intense in the TGCs under both in situ and in vitro conditions. The NADPH-diaphorase staining remained positive after fixation, which emphasizes the specific nature of the reaction, since fixation apparently blocks the NO formation ability of NOS but not its ability to transfer electrons from NADPH to reduce NBT [40,42,43]. This specificity was again reinforced as diaphorase staining became significantly decreased in the presence of L-NAME, an inhibitor of NOS, which prevents the conversion of L-arginine to NO [47].

Cultured trophoblast cells also exhibited diaphorase staining, but the number of reactive cells seemed to decrease during the culturing time. This may be due to the inability of the TGCs to maintain this enzymatic complex expression for long in vitro.

Immunodetection of both eNOS and iNOS isoforms in the postimplantation trophoblast cells supports the observations on NADPH-diaphorase activity. Similarly, coexpression of NADPH-diaphorase activity and endothelial and macrophage NOS has been described in the endovascular trophoblast in guinea pigs [48]. In our study, iNOS

was colocalized with eNOS in the trophoblast cells, and both reactions predominated in the cytoplasm of TGCs. The immunostaining pattern for the two isoforms was not identical, however.

Particularly in the case of TGCs from the EC, eNOS seemed to be preferentially concentrated on the surface of the trophoblast cells, as similarly reported by Iida and coworkers, in human placentas in the third month of pregnancy [49]. In contrast, iNOS occurred as patches in the cytoplasm of in situ TGCs and in a granular pattern throughout the cytoplasm in 48- and 72-h-cultured trophoblast cells. The reasons for these results are not clear but may reflect the presence of specific cytoplasmic areas associated with NO production (either membrane bound or not).

Conversely to our findings, some investigators of rodents have detected only iNOS in placental cells, in the late stages of pregnancy [50,51], whereas others [9] were unable to immunochemically detect NOS isoforms in near-term placenta cells. To reinforce the presence of NOS proteins in the trophoblast cell cytoplasm, ectoplacental extracts were submitted to SDS-PAGE. The identification of 130- and 135-kDa proteins suggested once again the presence of postimplantation trophoblast NOS isoforms that, on Western blot, reacted with polyclonal antibodies against iNOS and eNOS, respectively. The molecular mass of eNOS also coincides with the NOS isoform previously identified in other work using human placenta [52,53] or mouse implantation sites [50]. Interestingly, like Purcell et al. [50], we found in the Western blot two extra bands, with molecular masses below 130 kDa, reacting with anti-NOS antibodies. These authors suggested that this might result either from the expression of another NOS isoform or from a posttranslational modification of NOS [50]. A similar situation may be occurring in the mouse implanting trophoblast, although we have not investigated these possibilities.

We also estimated NO production by measuring nitrite using the Griess reaction. It is important to note that the use of EC cultures in these studies has allowed us to evaluate the product of NOS activity specifically resulting only from trophoblast cells, without influence of NO generated by leukocytes or decidual and metrial cells [37]. Our results strongly suggest that the implanting trophoblast has the ability to produce NO molecules. The drastic decrease in nitrite levels in the presence of L-NAME reveals that the conditioned medium of trophoblast cells must contain significant amounts of NO resulting from NOS activity. The type of enzymatic isoform source was not investigated; however, the significant increase in NO in the supernatants in the presence of γ-IFN favors the hypothesis of the presence and predominance of an inducible isoform. These data are consistent with the results of recent studies in rats and mice showing an increase of iNOS expression during pregnancy [37,40,50,51,54–56]. The expression of iNOS has also been found in the human placenta, particularly in Hofbauer cells, which are apparently involved in a specific cytostatic/cytotoxic effect against either immune challenges or pathogens present in the maternal organism [7]. In this context, during mouse embryo implantation, the activation of iNOS may be related to the intense phagocytic activity exhibited by the TGCs, and to the maternal regulatory mechanisms involved in this process [57,58].

Additionally, during this period of pregnancy, NO released to the maternal blood flow by trophoblast eNOS may also contribute to the success of the implantation process, helping to maintain vasodilatation and to prevent the coagulation of platelets on the trophoblast surface during the invasion process. Furthermore, the production of NO associated with superoxide anion generation by the mouse trophoblast [17,18] may form peroxynitrite [32], which in consequence may also lead to alterations to the vascular reactivity and the regulation of placental blood flow.

In conclusion, our data support the specific participation of the trophoblast in NO production during the postimplantation phase, which may represent mechanisms of fundamental physiological importance for the success of the early pregnancy.

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