

## **10. Immunomodulators in Human Trophoblast-Uterus Cross Talk: Cytokines, growth factors and nitric oxide**

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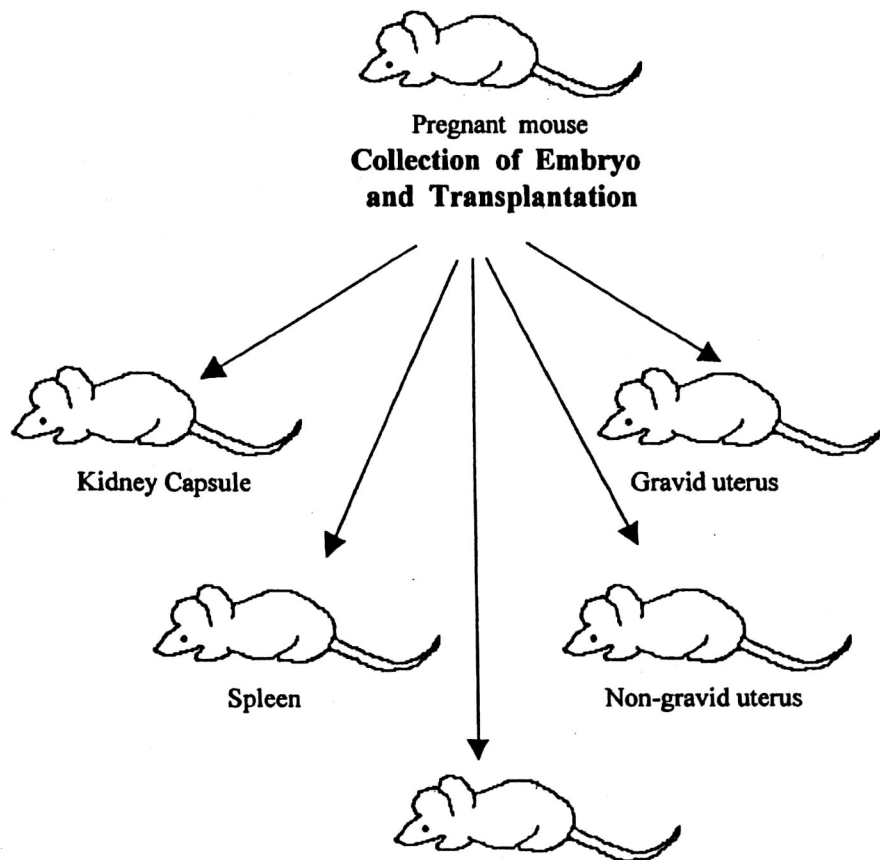
### **Abstract**

Successful reproductive outcome requires fine interplay of genetically dissimilar feto-placental unit, uterus and the local immune system. The role of immunomodulators elaborated by all the three has become increasingly apparent in the materno-fetal cross talk. Several of them, initially thought to be involved in pure immunological function, now seem to be of prime importance as determinants of optimal placental growth and function. These factors include growth factors, inflammatory and proinflammatory cytokines. Another molecule, nitric oxide, also involved in inflammatory responses, elaborated by macrophages and vascular endothelial cells, is now known to be synthesised by uterus and might have role in materno-fetal cross talk. To address the role of these cytokines and nitric oxide (NO) on optimal placental growth and function, we have studied their effect on trophoblast proliferation. Primary trophoblast cells, isolated from human term placentae and JEG-3 cells—a human trophoblast derived choriocarcinoma cell line, were used as *in vitro* models to assess the modulatory roles of cytokines and NO by metabolic labelling assay (MTT), Ki67 expression and BrdU incorporation. The results indicate that all the cytokines studied induced significant proliferation of trophoblast cells. Interestingly, maximum proliferation was obtained with inflammatory cytokines IL-1 $\alpha$ , IL-6 and TNF- $\alpha$  and NO donors. A mechanism of cytokine and NO induced trophoblast proliferation is being hypothesised.

### **Introduction**

The immune and reproductive systems although, have been considered independent of each other, the co-operation of both the systems are crucial for the initiation and maintenance of mammalian pregnancy. Implantation of the blastocyst to the endometrium is the first step in the initiation of successful pregnancy. The process can be viewed as a series of distinct events, many of which are similar to that of an inflammatory reactions and involves interactions between the trophoblast and the endometrium. The first attachment of the developing embryo to the uterus occurs through the trophoblast cells present in the outermost cell layer covering the blastocyst. Subsequently, the trophoblast cells breach the uterine epithelium and penetrate the basement membrane and the underlying connective tissue to form connection with the maternal circulation. In the utero-placental context these events of invasiveness

and proliferation of trophoblast cells are precisely controlled. The factors that contribute to this control mechanism are still largely unknown and pose serious question in the understanding of the biology of implantation. The classic experiments conducted by Kirby in early sixties (1960, 1963a, 1963b, 1965) in which mouse blastocysts were transplanted at several protected places like beneath kidney capsule, spleen, cryptorchid testis, scrotal testis, nongravid uterus and anterior eye chamber revealed that invasion of blastocyst in the adjoining tissue was always greater in these areas



Results (Kirby, 1963-1965)

Site	Implantation
Kidney Capsule	++
Spleen	++
Scrotal Testis	++
Cryptorchid	++
Non-gravid uterus	+++
Gravid uterus	Normal

Fig. 1. Implantation of blastocyst in different site of mouse and their relative implantation status.

where blood circulation was limited as compared to their invasion in the gravid uterus (Fig. 1). His elegant experiments indicated for the first time that the invasive property of blastocyst is an inherent one and the gravid uterus provides the regulatory switches to keep check on trophoblast invasiveness and proliferation.

Trophoblast cells are constantly exposed to uterine fluid rich in growth factors cytokines and hormones. These molecules may come from three different sources, the uterus, trophoblast cells themselves or the cells of local immune system. Often acting in groups, they regulate the uterine microenvironment via intercellular signals (Ben-Rafael and Orvieto, 1994). Three major cytokines critical for inflammatory responses,  $TNF\alpha$ , IL-1 and IL-6 are expressed in the endometrium and appear to serve various functions (Psychoyos et al., 1995). Stroma and epithelium express IL-1 $\alpha$  distinctly and IL-1 $\beta$  weakly. Estrogen and progesterone present in the uterine microenvironment influence the expression and the activity of cytokines such as IFN- $\gamma$ , GM-CSF, IL-6,  $TNF\alpha$ ,  $TNF\beta$  and IL-1 $\beta$  in the endometrium (Tabibzadeh et al., 1995). In addition, progesterone stimulates EGF and TGF- $\alpha$  expression in spiral arteries.

At the time of hatching of the blastocyst, human cytotrophoblasts partly begin to differentiate into syncytiotrophoblasts and produce increased amounts of hCG. As implantation approaches, it also starts synthesizing many growth factors, inducers, cytokines and paracrine factors such as insulin-like growth-factor (IGF), transforming growth factor (TGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF, Harvey et al., 1995). *In vitro* cultures of human blastocyst are shown to synthesize TGF $\alpha$  and IGF-II (Hemmings et al., 1992; Carson et al., 1993). Preimplantation mouse embryos express colony-stimulating factor (CSF-1) and the ligand for the proto-oncogene c-fms (Cullingford and Pollard, 1994). They also synthesize receptors for insulin, PDGF, TGF $\alpha$ , EGF, IL-1 $\beta$ , IL-1 and soluble IL-1 receptor antagonists (IL-ra) (Simon, 1994). Trophoblast cells seem to produce interferons (IFN) during early pregnancy. All the four subclasses of IFN, i.e.,  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\Omega$  are synthesized by trophoblast cells. These IFNs modulate the local lymphocyte proliferation and probably the trophoblast functions as well.

Implantation resembles the inflammatory reaction in many aspects. For example, increased blood flow at the site of implantation, and recruitment of lymphocytes at the same site. These lymphocytes and other cells of immune system secrete several protein factors which eventually participate in uteroplacental physiology. Uterine macrophages secrete cytokines IL-1,  $TNF\alpha$  and IL-1ra. IFN produced by local T cells seem to regulate cytokine activity and local cell-cell interactions, perhaps via the mediation of prostaglandinE2 and the expression of human leucocyte antigen (HLA-DR). Mouse and human placental tissue contain a large number of mononuclear phagocytes and these cells are the source of a IL-1 (Flynn et al., 1982). Activated T cells release IFN- $\gamma$ , which may induce HLA-DR expression in epithelial cells and inhibit those that do not express it.

Placenta-derived nitric oxide (NO) has been proposed to play a role in the regulation of feto-maternal blood flow (Buttery et al., 1994). Recent studies of Myatt et al. (1997) indicate its involvement in surveillance against maternal immune insult. The role of NO in trophoblast function is however, largely unknown. Since, trophoblast proliferation is the major cellular event in placental development, it could be

conjectured that NO may be involved in trophoblast proliferation like in enterocytes (Konturek et al., 1993) and glial cells (Munoz-Fernandez and Fresno, 1993).

In order to understand the role of cytokines and nitric oxide elaborated by uterine tissue, trophoblast cells and the uterine immune cells on trophoblast function, their effect on trophoblast cells was studied. Primary human trophoblast cells and a choriocarcinoma cell lines, JEG-3 were used in this study. Understanding of the biochemical mechanism(s) of control of trophoblastic invasion and proliferation may lead to the realization of the etiology of invasive carcinoma, metastasis and pathogenesis of trophoblastic invasive disorders.

## Materials and Methods

### Cell Culture

Human term placentae obtained from spontaneous vaginal delivery of healthy women were collected in normal saline and processed aseptically. Villous tissues were sampled from several cotyledons and rinsed extensively in normal saline to remove excess blood. Cells were isolated by differential trypsinization (0.125 g% of trypsin 1:250, Life Technologies, USA and 0.01% DNase, Sigma, USA) for  $3 \times 30$  minutes in calcium, magnesium free Hank's Balanced Salt Solution (CMF-HBSS) containing penicillin (100 IU/ml) and streptomycin (100  $\mu$ g/ml). The heterogeneous cell population was subjected to discontinuous Percoll (Pharmacia, Sweden) gradient (5–70%) centrifugation. The band corresponding to the density of 1.048–1.058 was 95% pure trophoblast cells (Kilman et al., 1986). These cells were plated onto 96-well tissue culture plates (Costar, USA) in DMEM-HG (Life Technologies, USA) containing 10% foetal bovine serum and the antibiotics. The plates were maintained at 37°C in sterile humid atmosphere. For cytochemical and immunocytochemical studies, cells were cultured on 12 mm coverslips.

JEG-3 cell line procured from American Type Culture Collection, USA were expanded in tissue culture flask in Ham's Nutrient mixture F12 (Life Technologies, USA) with 15% FCS and the antibiotic/antimycotic solution. Cells were harvested by trypsinization (0.25 g% trypsin, 0.03 g% EDTA in PBS), counted and plated onto 96 well plates for proliferation assay and in 12 mm coverslips for cytochemical and immunocytochemical studies.

### MTT Conversion Assay

The nonradioactive, colorimetric assay system for cell growth and viability using MTT was first described by Mosmann et al., (1983) The method was improved in subsequent years by several investigators for the measurement of cell proliferation in response to growth factors and cytokines (Tada et al., 1986; Denizot and Lang 1986; Vistica et al., 1991). The assay is based on conversion of MTT 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) salt by cellular dehydrogenases to insoluble formazan crystal and subsequent solubilisation of this formazan to produce colour which is directly proportional to the number of cells.

For MTT assay  $1 \times 10^5$  primary trophoblast cells or  $5 \times 10^3$  JEG-3 cells were plated in 96 well plates. Cells were challenged with different concentrations of growth factors and cytokines or with freshly prepared sodium nitroprusside (SNP)—a nitric

oxide donor, dissolved in medium or different concentrations of cytokines as mentioned in Table 1, such that the total culture volume for each well was 100  $\mu$ l. The concentrations of different growth factors and cytokines used are summarized in Table 1. After 48 hours of challenge, 10  $\mu$ l of MTT reagent (Boehringer Mannheim) was added to each well according to manufacturer's instruction. Four hours after addition of MTT, 100  $\mu$ l of solubiliser was added to each well. Plates were kept overnight in humidified chamber with 5% CO<sub>2</sub>. Absorbance was read by microtiter plate-reader (Anthos HT-II) at 550 nm taking 690 nm as reference filter. Data were expressed in terms of percent of control.

**Table 1. Summary of proliferative capacity of different growth factors**

Factor/Donors	Concentration range	% increase in proliferation	
		Term trophoblast	JEG-3
EGF	1–100 ng/ml	127–223	103–225
bFGF	0.1–10 ng/ml	113–439	134–608
TNF $\alpha$	50–5000 U/ml	133–181	117–360
TGF- $\beta$ <sub>1</sub>	0.1–10 ng/ml	122–170	141–677
TGF- $\beta$ <sub>2</sub>	0.001–5 ng/ml	103–221	121–479
IL-1 $\alpha$	5–500 pg/ml	112–339	117–203
IL-6	1–100 pg/ml	116–203	102–166
SNP	0.625–40 $\mu$ M		100–160

#### **Ki67 Index**

Cells grown on coverslips were transferred to 24 well cell culture plate and challenged with different cytokines or SNP for 48 hours. The concentrations of cytokines and SNP are mentioned in Table 2. Doses for Ki67 index were selected on the basis of the effects observed in MTT assay. Concentrations showing half-maximal induction of proliferation were used in these experiments. Coverslips were washed 3 times in phosphate buffered saline (PBS) and fixed in pre-chilled acetone at –20°C for 10 min. After fixation, coverslips were washed in PBS and processed for Ki67 immunolabelling. Briefly, non specific sites were blocked with normal horse serum and then incubated with mouse monoclonal anti-human Ki67 antibody. The antigen-antibody complex was visualized with Vectastain ABC kit followed by peroxidase staining using DAB as substrate. Finally, Ki67 positive cells over the total number of cells present were counted. In randomly selected fields a total of 2000 cells were counted per coverslip and Ki67 index was expressed as % positive cells.

#### **BrdU Incorporation and Flowcytometry**

BrdU (5-bromodeoxyuridine) incorporation followed by use of anti-BrdU monoclonal antibody to study DNA replication was first introduced by Gratzner (1982). Dolbere et al. (1983) devised a flowcytometric method for measurement of total DNA content using propidium iodide and incorporated BrdU to study cell proliferation. This method was further improved by Darzynkiewicz (1994) and had been used in our experiments with minor modification to study the effect of different growth factors, cytokines and SNP on JEG-3 cell proliferation.

Cells grown on 25 mm<sup>2</sup> tissue culture flasks or on coverslips were challenged with appropriate factors with same concentrations as mentioned for Ki67 index study. After 48 hours of challenge, the medium was removed from the flask, the cells were washed once with fresh medium, replaced with fresh medium and incubated with 30 µg/ml BrdU for 1 hour at 37°C in humidified atmosphere with 5% CO<sub>2</sub>. Finally, cells were harvested by trypsinization and dispersed thoroughly by repeated pipetting. The coverslips were removed from the medium and processed for BrdU immunocytochemistry. The antigen-antibody complex was visualized with FITC conjugated secondary antibody. The cell suspension was transferred to 1.5 ml microfuge tube and briefly centrifuged. The supernatant containing trypsin solution was discarded. The cell pellet was washed twice with 0.1 M PBS and fixed with 4% paraformaldehyde in 0.1 M PBS for 1 hour at 4°C. Non specific sites were blocked with 10% NHS containing 0.5% Triton X-100 in 0.1 M PBS for 30 min., following which the cells were incubated with 100 µl of undiluted anti-BrdU antibody for 1 hour at room temperature according to the manufacturer's instruction. At the end of the incubation with the primary antibody, cells were washed thrice with PBS and incubated with FITC-conjugated horse anti-mouse IgG at 1:200 dilution for 30 minutes at room temperature. After washing, cells were incubated with 10 µg/ml propidium iodide containing 100 U RNase A for 30 min at room temperature. They were then washed 3 times with PBS and resuspended in 1 ml PBS. The cell associated fluorescence was acquired in a Coulter FACS® apparatus. A total 10,000 cells were acquired. Data analysis and graphics generation was done using WinMDI software. Data was expressed in terms of mean peak value.

## Results

### Effect of Growth Factors, Cytokines and Nitric Oxide on Trophoblast Proliferation

Table 1 summarises the effect of different growth factors and cytokines on proliferation of primary trophoblast cells and JEG-3 cell line.

At doses ranging from 1–100 ng/ml EGF, primary trophoblast cells showed a dose dependent increase in proliferation. Maximum proliferation was observed at 20–100 ng/ml concentrations (160% to 223% of control). JEG-3 cells also showed dose dependent increase of proliferation but only at higher concentration of 20–50 mg/ml. Maximum proliferation of JEG-3 was observed at the dose of 50 ng/ml (225% of control). At the highest concentration of 100 ng/ml (212% of control) these cells showed less proliferation than 50 ng/ml. At lower concentrations of EGF the JEG-3 cells showed a variable response.

Increased proliferation of both primary and JEG-3 cells were observed with bFGF in a dose dependent manner at concentrations ranging from 0.1 to 10 ng/ml. As compared to EGF, bFGF exerted more pronounced effect on proliferation both in primary and JEG-3 cells with maximal proliferation observed at 10 ng/ml (438% and 608% respectively).

TNF $\alpha$  induced proliferation of JEG-3 cells only at higher concentrations ranging from 1000 to 5000 U/ml. In primary trophoblast cells, lower concentrations of TNF $\alpha$  ranging from 50–1000 U/ml induced only 20–30% proliferation. At higher doses of

2000 and 5000 U/ml a significant increase in proliferation was observed. The increased proliferation effect of  $TNF\alpha$  was much more pronounced in choriocarcinoma cells (JEG-3) than the primary trophoblast cells.

$TGF-\beta_1$  induced proliferation was also higher in JEG-3 cells than term trophoblast cells. There was a dose dependent increased proliferation in both the cell types.

Primary trophoblast cells responded with significantly increased proliferation at concentrations ranging from 0.2–5 ng/ml of  $TGF-\beta_2$ . Similar observations were made with JEG-3 cells as well.

$IL-1\alpha$  significantly induced primary trophoblast and choriocarcinoma cell proliferation. In JEG-3 cells,  $IL-1\alpha$  increased proliferation through all the range (10–500 pg/ml) with maximum proliferation at 500 pg/ml (203%). However, the response was not so prominent in primary trophoblast cells where lower concentrations of 5–50 pg/ml did not induce any proliferation. Concentrations higher than 50 pg/ml  $IL-1\alpha$  significantly increased proliferation of primary trophoblast cells.

$IL-6$  exerted almost similar effect on both the cell types. Maximum proliferation was observed at a dose of 100 pg/ml with 203% and 166% for primary trophoblast and JEG-3 cells respectively.

Increasing concentrations of SNP, a nitric oxide donor was observed to increase the proliferation of JEG-3 cells in a dose dependent manner as measured by MTT conversion assay. At doses from 2.5 to 40  $\mu M$  SNP, the mean values were significantly higher from the control. The fact that even at the highest concentration of 40  $\mu M$  a proliferation of about 160% was observed not only indicates its proliferation inducing ability but also shows that the compound is not cytotoxic.

#### Effect of Growth Factors, Cytokines and Nitric Oxide on Ki67 Expression

Ki67, a non-histone nuclear antigen present in  $G_1$ ,  $S$  and  $G_2$  but not  $G_0$  phases of cell cycle is one of the most widely used for analysis of cell cycle. To further substantiate the results obtained from MTT assay, Ki67 index was used to study the effect of the growth factors and cytokines on cellular proliferation of JEG-3 cells. All the growth factors and cytokines that increased proliferation in the MTT assay increased Ki67 index also. The results are shown in Table 2. The cytokines  $IL-6$ ,  $IL-1\alpha$ ,  $TNF-\alpha$  strongly induced Ki67 expression, the index being 12.40, 11.82 and 11.42 respectively as compared to the control index of 4.4. Other factors, e.g. EGF,

**Table 2. Effect of different growth factors and cytokines on proliferation index (Ki67) of JEG-3 cell**

Factors/Donors	Concentration	Ki67 Index
Control	plain media	4.40
EGF	50 ng/ml	5.10
$IL-6$	20 pg/ml	12.40
$IL-1\alpha$	200 pg/ml	11.82
$TNF\alpha$	2000 U/ml	11.42
$TGF-\beta_1$	2 ng/ml	7.61
$TGF-\beta_2$	2 ng/ml	6.46
bFGF	2 ng/ml	5.91
SNP	10 $\mu M$	7.2

TGF- $\beta_1$ , TGF- $\beta_2$  and bFGF significantly increased Ki67 expression but to a lower extent. It was observed that SNP also increased the expression of ki67 as compared to. In presence of 10  $\mu$ M SNP percentage of Ki67 positive cells increased from 4.4 to 7.2.

### Effect of Growth Factors, Cytokines and Nitric Oxide on BrdU Incorporation

To further substantiate the results of MTT assay and ki67 index, BrdU incorporation followed by immunofluorescence was used to study the proliferation effect of different factors on JEG-3 cells. The values obtained after counting BrdU positive cells over total cells under fluorescent microscope is summarized in Table 3, which indicates that IL-6, IL-1 $\alpha$  and TNF $\alpha$  exerted maximum effect on BrdU incorporation. Other factors also increased BrdU incorporation significantly but to a lower extent. Though, the pattern of proliferation results remained the same as that obtained with Ki67, the values were lower in the case of BrdU incorporation. SNP also increased the BrdU incorporation in JEG-3 cells. The mean fluorescence of control cells was 26.41 which increased to the level of 68.87 after challenge with SNP.

**Table 3. Effect of different growth factors and cytokines on BrdU incorporation by JEG-3 cells**

Factors/Donors	Concentration	BrdU incorporation (% of cells)	Mean fluorescence intensity
Control	plain media	2.33	26.60
EGF	50 ng/ml	3.40	45.09
IL-6	20 pg/ml	7.20	71.20
IL-1 $\alpha$	200 pg/ml	8.20	72.38
TNF $\alpha$	2000 U/ml	6.70	66.65
TGF- $\beta_1$	2 ng/ml	6.00	58.45
TGF- $\beta_2$	2 ng/ml	5.50	61.09
bFGF	2 ng/ml	5.20	56.43
SNP	10 $\mu$ M	6.50	68.87

### Discussion

During early embryo development and subsequent placentation, the trophoblast cells are bathed in uterine fluid rich in growth factors and cytokines. These agents are shown to regulate proliferation and differentiation of a variety of cell types depending on the microenvironment. Since, very little is known about the molecular signals that regulate proliferation of trophoblast cells, studies were undertaken to investigate the effect of different growth factors and cytokines on proliferation of cytotrophoblast cells. The differentiated multinucleated syncytial cells are nonproliferative. When mononucleated cyto-trophoblast cells were challenged with EGF, bFGF, TNF $\alpha$ , TGF $\beta_1$ , TGF $\beta_2$ , IL-1 $\alpha$  and IL-6, all induced proliferation of both primary cytotrophoblasts as well as JEG-3 cell line to varying degrees as measured by MTT conversion assay (Table 1). Among all the ligands used, the trophoblast cells were found to be more sensitive to bFGF, TGF $\beta_1$  and TGF $\beta_2$ . Not only these three factors induced proliferation at a low concentration but also brought about much higher



degree of proliferation (Table 1). The Ki67 index and BrdU incorporation data also substantiated the findings obtained with MTT conversion assay. The proliferation index (Ki67) and mean fluorescence intensity obtained with these two assays were however, highest with IL-6, IL-1 $\alpha$  and TNF $\alpha$ . Some of these findings are quite contrary to the ones reported earlier, especially the stimulation in proliferation observed with TGF $\beta$  and TNF $\alpha$ . In the present study, not only a systematic study with varying concentration of the factors was carried out but the assay used to detect the proliferation was a very sensitive one. The findings were later corroborated by two other systems used for detecting proliferation of the cells. It could be possible that the same ligands may induce proliferation or differentiation depending on the concentration of the ligand and receptivity of the cell to these ligands. This may explain the reasons for having contradictory reports with the same ligand e.g. EGF has been reported to induce proliferation (Maruo et al., 1992) and also shown to have no effect on trophoblast proliferation by others (Morrish et al., 1987).

In the placental tissue, trophoblast cells are surrounded by endothelial cells as well as macrophages, all of which express different isoforms of NOS leading to the local generation of NO. To address the role of NO on trophoblast function, we have further studied the effect of NO using SNP as the NO donor on trophoblast cell proliferation. NO released from SNP significantly stimulated the proliferation of JEG-3 cells over and above their normal rate of proliferation in culture (Table 1). SNP induced proliferation of JEG-3 cells as measured by increased metabolic activity (MTT conversion) was further confirmed by induction of Ki67, a proliferation associated nuclear protein (Table 2). Proliferation-associated increased DNA synthesis as evaluated by BrdU incorporation also supports the observed SNP enhanced proliferation (Table 3). Taken together, these observations indicate that SNP through the generation of NO promotes proliferation of trophoblast cells.

Indeed, in recent years, nitric oxide has been proposed as an intracellular messenger for cellular proliferation and differentiation (Garg and Hassid 1990; Garg et al., 1992; Yang et al., 1994; Konturek et al., 1993; Munoz-Fernandez and Fresno, 1993; Ziche et al., 1994; Peunova and Enikolopov, 1995). The enzyme responsible for the synthesis of NO is localized in the placenta as well as in the uterus. In the present study, SNP induced enhancement of proliferation of trophoblast cells which may be another example of the capacity of NO to modulate proliferation. NO may induce opposite effects in other cells like in myoblast (Lee et al., 1994) and neuronal cells (Peunova and Enikolopov, 1995), it enhances differentiation, whereas in bone marrow derived cells, it increases proliferation (Shami and Weinberg, 1996). Our studies indicate that NO induces proliferation of human trophoblast cells. The mechanism by which such a control may be exerted needs to be further elucidated.

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