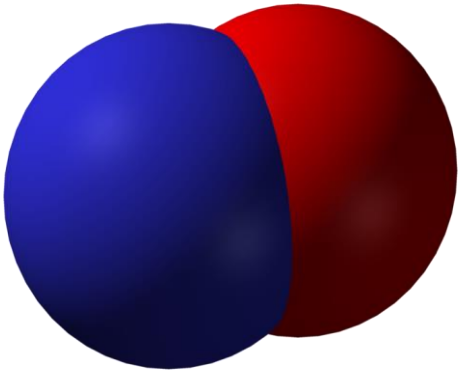
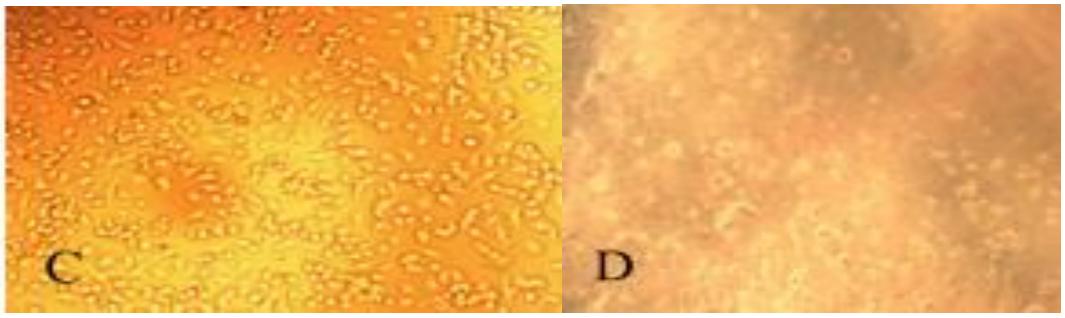
Nitric oxide production - a comparison between macrophages and trophoblasts under conditions of induced inflammation





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5.- årsoppgaven – Profesjonsstudiet i medisin ved UiT Norges arktiske universitet

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Sammendrag

En vellykket implantasjon av en blastocyst i veggen av uterus kan lede til graviditet, og en mislykket implantasjon vil føre til tap av graviditet. Dersom dette skjer gang etter gang blir resultatet i praksis infertilitet. Implantasjon av blastocysten i veggen av uterus kan oppnås ved den naturlige metoden eller ved assistert befruktning (In Vitro Fertilisasjon). Mange hormoner, cytokiner, kjemokiner og andre molekyler spiller en viktig rolle ved denne prosessen. Nitrogen oksyd (NO) kan være en slik faktor som antas å fremme og/eller hemme implantasjonsprosessen. Effekten avhenger av type celler (trofoblaster eller makrofager) som produserer NO og kvantiteten som blir produsert. Mitt mål var å kvantifisere NO produsert av makrofager og trofoblaster ved indusert inflammatoriske tilstander.
Lipopolysakkarid i konsentrasjon 0-20 µg/mL ble brukt for å indusere inflammatorisk NO produksjon i både makrofager og trofoblaster. Nitritt, et stabilt sluttprodukt av NO ble målt ved hjelp av Griess reagens og spektrofotometriske analyse, etter 8 og 24 timer.

Produksjon av NO i makrofagene var signifikant, men trofoblastene visste ingen tegn til NO produksjon. Det betyr at under inflammatoriske tilstander produserer makrofager mer NO enn trofoblaster, og dette kan muligens påvirke trofoblastene.

Abstract

Background: Implantation of the conceptus in the uterus, whether achieved naturally or via In Vitro Fertilization is a precondition for pregnancy. Its failure leads to a failure to conceive. Many hormones, cytokines, chemokines and other molecules play a role in the implantation process. Nitric oxide (NO) is said to be one of them. It may play an important role in determining success or failure in the implantation process, depending upon which cells (trophoblasts or macrophages) that produce NO and the quantity that is produced. [10]

Aims of the thesis: Trophoblasts and macrophages are important cells lines involved in the implantation process, and both produce nitric oxide. Trophoblasts play a role in successful penetration of the endometrium and macrophages regulates apoptosis of the trophoblasts.[8] Our aim was to determine the quantity of NO produced by macrophages and trophoblasts, under similar conditions and duration of induced inflammation, and to compare the two values.

Materials and methods: A murine macrophage cell line, J744.1 and a trophoblast (HTR-8/SVneo) cell line, were cultured, induced by similar lipopolysaccharides (LPS) concentrations (0 to 20 μg/mL), to initiate inflammatory nitric oxide production. Nitrite, a stable end product of nitric oxide, was measured by adding Griess reagent and carrying out spectrophotometric analysis.

Results and conclusion: Macrophage nitrite values increased many fold in the first 8 hours and from 8 to 24 hours. For lipopolysaccharide concentration of 2 μg/mL, macrophages nitrite values, from 8 to 24 hours increased significantly. Trophoblast showed no nitrite production after 8 hours and no significant nitrite values were recorded, from 8 to 24 hours. Trophoblast nitrite values did change to positive from 8 to 24 hours, but remained insignificantly low.

In other words, we observed that macrophages produced significant amounts of nitric oxide, while there was no nitric oxide production in trophoblasts under identical conditions. This suggests that macrophages produce more NO under induced inflammatory conditions than trophoblasts and this may affect trophoblasts by regulating apoptosis of the trophoblasts.

Acknowledgements

I would like to express my gratitude to my supervisor, Professor Knut Tore Lappegård, Co-supervisor Sandy Goldbeck-Wood and my previous supervisor Purusotam Basnet for help, support and guidance.

My previous supervisor Purusotam Basnet and I carried out two successful experiments, where I played the role of assistant for the most part of the experiment. Sometimes I worked independently, according to instructions given by the supervisor like cell counting by using hemocytometer, preparing LPS solution or carrying our spectrophotometric analysis. Our one successful experiment with macrophages came after two failed attempts to grow macrophages. Twice macrophages failed to grow and we had to restart everything from scratch. Because of these failed attempts, which were both time consuming, we limited our experiments to one successful experiment with macrophages and one with trophoblasts. We were uncertain of the reasons for these failures but contamination of the cells at some point is the most likely explanation. In our third attempt we were more careful in every respect and successfully carried out the experiment.

Zafar M. Alam

May, 2014

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Nitric oxide figure in the title page is from Wikipedia. [22]

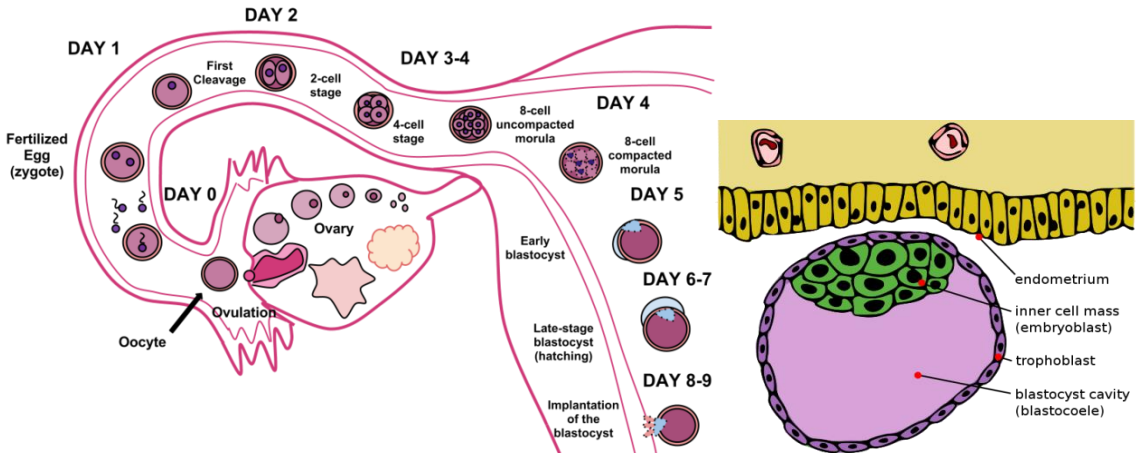
List of abbreviations

Abs	Absorbance
Ca^{2+}	Calcium
CI	Confidence Interval
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelium nitric oxide synthase
FCS	Fetal calf serum
H_3PO_4	Phosphoric acid
ICSI	Intracytoplasmic sperm injection
IL	Interleukin
INF-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
IVF	In Vitro Fertilization
L-NAME	N^G-nitro-l-arginine methyl ester
LPS	Lipopolysaccharides
M	Macrophages
$NaNO_2$	Sodium Nitrite
NED	N-1-naphthylethylenediamine dihydrochloride
NO	Nitric oxide
NO_2^-	Nitrite ion
NOS	nitric oxide synthase
RT-PCR	Reverse-Transcription-Polymerase Chain Reaction
SD	Standard deviation
SE	Standard error
STD	Standard solution
SUL	Sulphanilamide
T	Trophoblast
TGF	Tumor growth factor
TNF	Tumor necrosis factor

1.0 **Introduction**

Despite developments in modern medicine, we still have long way to go in solving the problems of infertility, failed pregnancies and congenital defects. Pregnancy is a process that begins with implantation of embryo into the wall of the uterus. Implantation is the process of adherence, invasion and penetration of the blastocyst into the wall of the uterus. Implantation is a very important event whether pregnancy is achieved naturally or by In Vitro Fertilization (IVF). Implantation achieved by natural process, is the sequence of events, leading to insertion of blastocysts into the wall of the uterine. Many complications and physical defects can lead to implantation failure, and loss of pregnancy. IVF is the commonly used treatment for the couples who fail to achieve pregnancy by natural means, and leads to implantation of blastocyst (mostly) into the wall of the uterus.

1.1 **Natural fertilization and implantation process.**



A B

Figure 1. *Fertilization and implantation. "A" shows different stages of fertilization process. "B" shows the blastocyst about to implant. Figures are from Wikipedia.* [23, 24]

During every ovulatory cycle, one dominant follicle emerges which ruptures, and releases the ovum.[1] The human male sperm undergoes the process of capacitation and the acrosome reaction before penetrating into the perivitelline space to attach to ovum plasma membrane. The plasma membranes of both ovum and sperm fuse, and the sperm enters the ovum to fertilize it.[2]

The process of fertilization leads to formation of a zygote (diploid, 46 chromosomes) which undergoes cleavage to become blastomere and then morula (depending on the numbers of cells).[3] The morula enters the uterine cavity. The blastocyst is formed by accumulation of fluid among cells of morula, and has an inner cell mass (embryoblast) and an outer cell mass (trophoblast). The initial adhesion of the blastocyst to the uterine wall is called apposition. Initial attachment, apposition and firm adherence involves a glycosylated protein (e.g. mucin), cell adhesion molecules (e.g. L-selectin), and integrins, and is regulated by paracrine interaction between the invading blastocyst and endometrium.[3] The successful penetration (by protease activity) and invasion of the endometrium occurs by differentiation of the invading trophoblast tissue into;

1) Syncytiotrophoblast – an outer layer.
2) Cytotrophoblast – an inner layer. Which further divides into;
   I) Villous trophoblast – (this gives rise to chorionic villi, which transport oxygen and nutrients)
   II) Extravillous trophoblast which further divides into:
      - interstitial trophoblast – (penetrates decidua, and myometrium)
      - endovascular trophoblast – (invades the smooth muscles wall of the spiral arteries).[3]

1.2 **Failure to implant and In Vitro Fertilization.**

In some couples, the above process fails, which leads to loss of pregnancy and infertility.[4] In Vitro Fertilization is a treatment that leads to implantation of blastocyst into the uterine wall. IVF may be a long or relatively short process in which eggs are retrieved from the ovary, fertilized in the laboratory, and replaced in the uterus. [5, 6]

1.3 **Nitric oxide (NO).**

The mechanism and the molecules which guide the implantation processes are not clearly understood, yet. Many factors like hormones, cytokines, chemokines and other molecules affect the process of implantation, directly or indirectly. Nitric oxide (NO) is one such factor influencing the implantation process, mentioned in many studies, but the extent of its influence is not fully clear. Nitric oxide is a water- and lipid soluble inorganic molecule, defined as;

["NO is a product of macrophages activated by cytokines, microbial compounds or both, is derived from the amino acid l-arginine by the enzymatic activity of inducible nitric oxide

synthase (iNOS or NOS2) and functions as a tumoricidal and antimicrobial molecule in vitro and in vivo."][7]

Table 1.

Physiological qualities of nitric oxide synthase (NOS) enzyme, its isomers, activation, quantity and duration of nitric oxide (NO) production, effects and regulation.[19, 20,21]

Isomers of NOS (nitric oxide synthase)	cNOS (Constitutive NOS)		iNOS Inducible nitric oxide synthase or type II, NOS-2
	nNOS Neuronal nitric oxide synthase or type I, NOS-1	eNOS Endothelium nitric oxide synthase or Type III, NOS-3	
Activated by	Influx of Ca^{2+}, it binds to Calmodulin receptors		Cytokines like INF-γ,TNF-α, IL-1 and LPS (endotoxin)
Quantity and duration of NO production	Low amount, Short duration		High/great amount, long duration
Predominant effects	Direct effect like metal complexes and high energy radicals dominates.		Indirect effects like nitrosation, nitration, and oxidation reaction dominates.
Inhibitors	● 7-nitro-indazole inhibits NOS-1, and is located in respiratory epithelium, Stomach and uterus (rats). ● Methylarginine (L-NAME) and Dimethylarginine inhibits NOS-3, located in epithelium and neurons.		●Aminoethyl-isothiourea located in chondrocytes and tumor cell (human) ●Aminoguanidine: drug, inhibits in cardiac myocytes. ● Glucocorticoids, TGF, IL-4, IL-10

L-NAME = N^G-nitro-l-arginine methyl ester

1.3.1 The role of Nitric Oxide in the implantation process

Nitric oxide is well known for its contradictory roles. The role of nitric oxide in a given situation depends on many factors i.e. the cell type, the concentration of nitric oxide produced, the duration of its production, and the signaling mechanism which mediates its effects.[8] Trophoblast expresses both the constitutive (eNOS) and the inducible isoforms (iNOS) of nitric oxide synthase, and nitric oxide produced by trophoblast plays a positive role in the process of implantation, as mentioned among other by Moraes and Kaufman. Moraes suggested that trophoblast cell motility and invasion strongly depend on trophoblast-derived NOS [9] and Kaufman showed that NO dilated uteroplacental arteries which were later invaded by trophoblast.[10] Macrophages, mostly present in the decidua basalis, produce NO to regulate trophoblast invasion[10] by regulating apoptosis in trophoblast [8] and may play a negative role in the process of implantation;

["However, combining both possible interactions between macrophages and the trophoblast, it could be hypothesized that macrophage-induced trophoblast apoptosis attracts and activates more macrophages, leading to a vicious cycle. In normal pregnancy, the walls of uteroplacental arteries are largely devoid of macrophages and become invaded by the trophoblast. In contrast, preeclampsia is associated with reduced trophoblast invasion of uteroplacental vessels, and accumulation of apoptotic interstitial trophoblast juxtaposing the arteries correlate with maternal macrophages in the arterial media."][10]

1.4 **Aim of the thesis**.

As seen from the literature, different studies attribute different roles to nitric oxide produced by trophoblast or macrophages in the process of implantation. Since both produce nitric oxide, the question is how much nitric oxide is produced by macrophages and how much nitric oxide is produced by trophoblasts and does the relative amounts produced in the two cell types offer us any clues as to their roles in successful implantation. The level of nitric oxide produced by maternal macrophages and the level of nitric oxide produced by trophoblast on the fetal side might each play an important role in embryo implantation. In this experiment we tried to measure the amount of nitric oxide produced by trophoblasts and macrophages under similar inflammatory conditions. Both cell lines were cultured by using similar type of media, induced by same concentration of LPS, and the amount of nitrite was measured in similar fashion.

2.0 **Materials and methods**

2.1 **Cell line**

Murine macrophage-like cell line, J744.1 and trophoblast (HTR-8/SVneo) cell line (From: Duke/UNC/UTA/EBI ENCODE group) were used, which display an unlimited life-span in culture, share features with invasive trophoblast such as expression of cytokeratin 18 and some EVT-specific integrins.[11]

2.2 **Chemicals and reagents**

Most of the material used was from Sigma Life Science (Sigma–Aldrich) such as RPMI-1640 Medium with sodium bicarbonate and L-glutamine, trypsin–EDTA (ethylenediaminetetraacetic acid) (T 3924) w/0.5 g profane trypsin and 0.2 g EDTA, lipopolysaccharide (LPS; *Escherichia coli*, 055:B5) (L2890-25 MG), Sulfanilamide, N-(1-Naphthyi) ethylenediame dihydrochloride (N 9125-10G), Phosphoric acid (H_3PO_4) in liquid form, $NaNO_2$ in solid form, and Fetal calf serum (FCS). Hemocytometer fromTiefe 0.100 mm, 0.0025 mm^2, Spectrophotometer from Agilent 8453 with ultra-micro 50 Cuvette.

2.2.1 Preparation of Griess Reagent

Two hundred mL of Griess reagent was prepared by adding 5 mL of Phosphoric acid (H_3PO_4) into a bottle containing 195 mL of distilled water, to make 2.5 % H_3PO_4 solution. Two grams (1 %) Sulphanilamide and 200 mg (0.1 %) N-1-naphthylethylenediamine dihydrochloride (NED) were dissolved slowly into 2.5 % H_3PO_4 solution, in ~ 4 - 5 minutes. A glass bottle containing Griess Reagent was wrapped into aluminum foil, labeled, and stored in a refrigerator.

Nitric oxide (NO), under physiological conditions is highly unstable and is oxidized into the more stable nitrite (NO_2^-) ion or trapped by thiols as an S-nitroso adduct. Griess reagent provides acidic conditions for nitrite ion to react with Sulphanilamide (SUL). This reaction produces a colorless Diazonium salt. Diazonium salt reacts with N-1-naphthylethylenediamine dihydrochloride (NED) to produce an Azo dye of purple colour.[12, 13]

$NO_2^- + H^+ + C_6H_8O_2N_2S \Rightarrow$ Diazonium salt + $C_{12}H_{14}N_2 \Rightarrow C_{18}H_{19}O_2N_5S + 2H_2O$

(Nitrite) (Acid) (SUL) (NED) (Azo dye) (Water) [13]

2.2.2 Standard solution of sodium nitrite ($NaNO_2$)

Standard solutions of $NaNO_2$ were prepared for the quantification of nitrite produced by the cells in the medium. As the molecular weight for $NaNO_2$ is 69, we started with mixing 69 g of $NaNO_2$ into 100 mL of water, to a get stock solution (see Table 2).

Table 2.

Different sample solutions of sodium nitrite.

Mixture	**Concentration of $NaNO_2$**	**Level**
69 g $NaNO_2$ + 100 mL of H_2O	10 mM (100 mL) $NaNO_2$	(0) Stock solution
1 mL (0) + 9 mL of H_2O	1mM or 1000 μM (10 mL) $NaNO_2$	(STD)
400 μL (STD) + 3600 μL H_2O	100 μM, (4 mL/) $NaNO_2$	(A)
2 mL (A) + 2 mL H_2O	50 μM, (4 mL) $NaNO_2$	(B)
2 mL (B) + 2 mL H_2O	25 μM, (4 mL) $NaNO_2$	(C)
2 mL (C) + 2 mL H_2O	12.5 μM, (4 mL) $NaNO_2$	(D)
2 mL (D) + 2 mL H_2O	6.25 μM, (4 mL) $NaNO_2$	(E)
2 mL (E) + 2 mL H_2O	3.12 μM, (4 mL) $NaNO_2$	(F)
2 mL (F) + 2 mL H_2O	1.56 μM, (4 mL) $NaNO_2$	(G)
2 mL (G) + 2 mL H_2O	0.78 μM, (5 mL) $NaNO_2$	(H)

Note. H_2O = Water, $NaNO_2$ = Sodium nitrite, STD = Standard solution.

All standard solutions and the Griess reagent were kept at room temperature for at least 2 hours. $NaNO_2$ solutions were mixed with equal volume of Griess reagent (350 μL each). They were left in the dark for 30 minutes and then spectrophotometric measurements were carried out at 550 nm wavelength. Each sample was measured in triplicate. A standard curve was obtained by plotting $NaNO_2$ solution concentration along the x-axis and average value of UV absorbance (Abs) along the y-axis.

2.3 **Preparation of the medium for the cell culture**

RPMI 1640 medium with sodium bicarbonate and L-glutamine in 500 mL bottle was purchased. Fifty mL of this RPMI 1640 medium was taken out and put into a 50 mL tube. The tube was labeled and stored in the refrigerator (for later use). Fifty mL FCS was then added into 450 mL RPMI medium bottle to make 10 % FCS medium. The bottle was shaken to

make the solution homogeneous and labeled. The cell culture medium for trophoblast (HTR-8/SVneo) cell line contained 5 % FCS (25 mL) instead of 10 % used for macrophages.

2.4 **Cell culture protocol**

The cells were taken out of liquid nitrogen. The flask with cells was wrapped with aluminum foil and thawed by vigorous rubbing in a warm palm. Some cells were then transferred into the culture flask (25 cm^2) containing pre-warmed culture medium (7 mL) with the help of 1 mL micropipette. The flask was then incubated at 37^oC, CO_2 = 0.50. After one hour, the medium contaminating cryopreservative (DMSO) was replaced very gently by pre-warmed culture medium (7 mL). The medium was then changed every 24 hours until a confluent monolayer of cells developed.

It was at this point that our macrophages failed to grow, twice. It took us three days to get there. We were uncertain of the reasons for why it happened, but the most likely explanation would be that there may have been contamination of cells at some point. We succeeded in growing macrophages in our third attempt.

2.4.1 Splitting of cells with trypsin

Eight mL of RPMI medium with FCS was added into each of the two culture flasks (75 cm^2) and both were put into incubator. The medium was removed (sucked out) from 25 cm^2 flask, and washed with 8 mL RPMI medium (without FCS). Pre-warmed 6 mL of trypsin, from Trypsin-EDTA bottle, was wadded to 25 cm^2 flask. The flask with trypsin was put into the incubator and warmed for ~ 3 - 5 minutes. The flask was then taken out, shaken, jerked and observed under the microscope. The cells were seen separated from each other. The trypsin containing cell suspension was then transferred into a 50 mL tube. The medium with FCS, in almost the same volume as that of trypsin was added (12 mL) to neutralize the effects of trypsin.

2.4.2 Centrifugation and cell suspension

The tube containing the cell suspension (50 mL) with medium and trypsin was put on the one side of the centrifuge and another 50 mL tube with water was put on opposite side of centrifuge (to balance). The centrifuge was set at 1100 rpm, for 5 minutes. After centrifugation, the supernatant was removed. Some medium was added and a homogeneous

suspension was prepared. The cell suspension was diluted to 40 mL with the medium, and 5 mL was transferred to each of the two 75 cm^2 flasks. Approximately 30 mL of cell suspension was diluted to 50 mL in a 50 mL tube, and adjusted to 5 x 10^5cells/mL after cell counting. Fifty mL tube was used in preparation of LPS solution.

2.4.3 Cell counting using Hemocytometer

One hundred μL cell suspension was added to 400 μL medium to create a dilution factor of 5, from an original volume of 30 mL.[14] A hemocytometer was used to count the cells. A hemocytometer has 9 large squares (grids) in one chamber.

Area of each large square is 1 mm x 1 mm = 1 mm^2. Each square has a depth of 0.1 mm. So each square represents total volume of 0.1 mm^3 (1 mm^2 x 0.1 mm) = 10^{-4} cm^3 (since 1 mm = 10^{-3} cm) = 10^{-4}mL (since 1 cm^3 = 1 mL) = 0.1 μL (since 1 mm = 10^3 μL, so 0.1 x 10^{-3} x 10^3) = 0.1 x 10^3 nL (since 1 μL = 10^3 nL) = 100 nL. The central grid (number 5) has 25 square, so each square has a volume of 100 / 25 = 4 nL.

The number of cells counted in the central grid (25 small squares) gave the total cell number in 100 nL which was calculated to cell number per mL and adjusted by the dilution factors to find out the correct cell number in the original tube.

2.4.4 Cell plating

After preparing the correct dilution of cell suspension, each 1 mL cells suspension was transferred into the two 24 wells culture plates.

2.5 **Preparation of LPS solution and treatment**

Lipopolysaccharide (LPS) solution (1 mg/mL equivalent to 1μg/μL) was prepared in the medium and diluted with medium from 50 mL tube (cell suspension), as follows:

In the first tube we put 10 μL of LPS + 9.990 mL of medium (10 mL) ----- 1μg/mL.

In the second tube we put 20 μL of LPS + 9.980 mL of medium (10 mL) --- 2 μg/mL

In the third tube we put 50 μL of LPS + 9.950 mL of medium (10 mL). ----- 5 μg/mL

In the fouth tube we put 100 μL of LPS + 9.900 mL of medium (10 mL) ---- 10 μg/mL

In the fifth tube we put 200 μL of LPS + 9.800 mL of medium (10 mL) ---- 20 μg/mL

After 24 hours, the cell (5 x 106 cells/mL) incubation, medium was replaced with fresh medium containing LPS as shown below:

Table 3.

LPS distribution in 24 wells culture plates.

Control	LPS-1	LPS-2	LPS-5	LPS-10	LPS-20
Control	LPS-1	LPS-2	LPS-5	LPS-10	LPS-20
Control	LPS-1	LPS-2	LPS-5	LPS-10	LPS-20
Control	LPS-1	LPS-2	LPS-5	LPS-10	LPS-20

Note. LPS = Lipopolysaccharide measured in μg/mL, Control = No LPS

One plate was incubated up to 8 hours and NO production by the cells in the medium was measured. The second plate was incubated up to 24 hours and measurements were made the same way as after 8 hours.

2.6 **Spectrophotometric analysis**

Quantitative analysis for nitrite was carried out after 8 and 24 hours incubation. From each well 350 μL medium was taken out and placed into the Eppendrof tube (1.5 mL). Twenty four such tubes were prepared, one for each well. In each tube, 350 μL of Griess reagents was added and allowed to react for 30 minutes. This reaction mixture was then transferred from 1.5 mL tube into a cuvette and the cuvette was put in the spectrophotometer, the wavelength was adjusted to 550 nm absorbance, and reading was carried out. The amount of NO produced by the cells is equivalent to the corresponding nitrite, which reacts with Griess reagent to give a pink color. With the help of a standard curve the amount of NO produced by the cells was expressed as μM NO production under the LPS challenge.

3.0 **Results**

3.1 **Sodium Nitrite ($NaNO_2$) solutions**

Sodium Nitrate solutions were used to produce the standard curve for nitrite absorbance (n = 1). For every concentration of $NaNO_2$ sample, three different absorbance values were collected. The mean and standard deviation (SD) was calculated for each.

Table 4.

Mean absorbance values for $NaNO_2$ solutions.

Concentration $NaNO_2$ (µM)	0.78	1.56	3.12	6.25	12.5	25	50
Average NO_2^- Absorbance	0.032	0.063	0.123	0.239	0.472	0.918	1.775
SD(s) ±	0.002	0.002	0.002	0.005	0.005	0.011	0.008
% change in Absorbance	0	95%	96%	94%	98%	95%	93%

Note. NO_2^- = Nitrite, $NaNO_2$ = Sodium nitrate, SD = Standard deviation,

Average NO_2^- absorbance values were used to produce a standard curve, as shown in Figure 2. It also shows fine linear line (Trend line) with value y = 0.0354 x +0.0154.

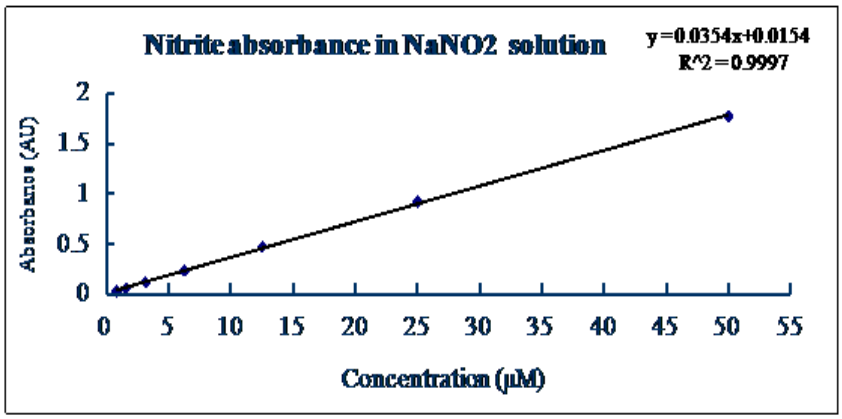


Figure 2. *Standard Curve for nitrite (NO_2^-) absorbance for $NaNO_2$ Solutions.*

3.2 **Macrophage NO production after 8 hours stimulation by lipopolysaccharide**

For each lipopolysaccharide (LPS) concentration, four different absorbance values were collected (n = 1). For example, the sample with LPS concentration of 1 μg/mL was put into four different cuvettes and these cuvettes were placed in the spectrophotometer to get four different absorbance values. The mean and standard deviation (SD) was calculated for each.

Table 5.

Mean NO_2^- absorbance values for macrophages after 8 hours.

LPS concentration (μg/mL)	0	1	2	5	10	20
Absorbance NO_2^-	0.034	0.05	0.044	0.046	0.046	0.045
SD (s) $\pm$	0.042	0.001	0.002	0.001	0.0003	0.001
% change in Absorbance	0	33%	-1%	4%	-1%	-3%
% change in LPS concentration	0		100%	150%	100%	100%

Note. LPS = Lipopolysaccharide, NO_2^- = Nitrite, SD = Standard deviation,

With the help of the standard curve shown in Figure 2, the amount of NO produced by the macrophages was expressed as μM NO production under the LPS challenge. NO_2^- absorbance values were put as y value in formula y = 0.0354 x +0.0154 and x= ((y - 0.0154)/0.0354) was calculated, as shown in Figure 3.

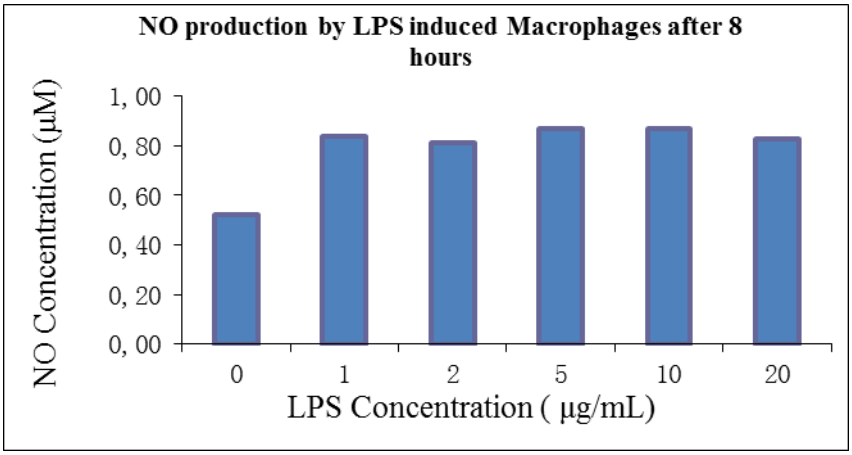


Figure 3. *Nitric oxide production by macrophages measured in μM after 8 hours stimulation with LPS.*

3.3 **Macrophage NO production after 24 hours stimulation by lipopolysaccharide** (Figure 6)

For each lipopolysaccharide (LPS) concentration, four different absorbance values were collected in a similar manner as for NO production in macrophages after 8 hours (n = 1). The mean and standard deviation (SD) was calculated for each.

Table 6.

Mean *NO_2^- absorbance values for macrophages after 24 hours.*

LPS concentration (μg/mL)	0	1	2	5	10	20
Average NO_2^- absorbance	0.0239	0.4087	0.4450	0.4600	0.4424	0.4121
SD (s) ±	0.006	0.006	0.014	0.025	0.021	0.009
% change in Absorbance	0	1606%	9%	3%	-4%	-7%

Note. LPS = Lipopolysaccharide, NO_2^- = Nitrite, SD = Standard deviation

With the help of the standard curve shown in Figure 2, the amount of NO produced by the macrophages was expressed as μM NO production under the LPS challenge. NO_2^- absorbance values were put as y value in formula y = 0.0354 x +0.0154 and x= ((y - 0.0354)/0.0154) was calculated, as shown in Figure 4.

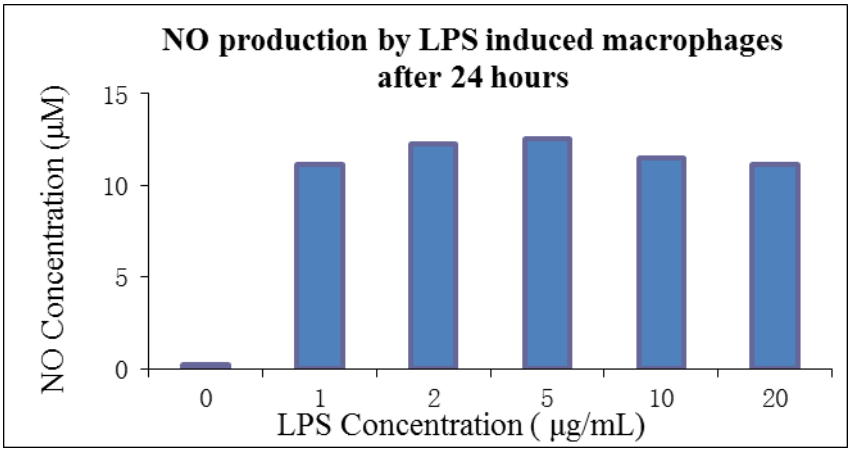


Figure 4. *Nitric oxide production by macrophages measured in μM after 24 hours stimulation with LPS.*

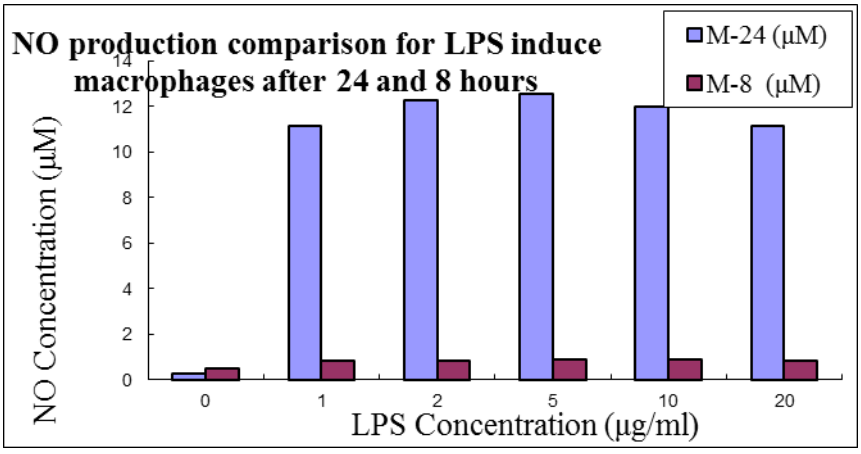


Figure 5. *Comparison of nitric oxide produced by the macrophages expressed as µM*

M-8 = Macrophages after 8 hours, values from figure 3.

M-24 = Macrophages after 24 hours, values from figure 4.

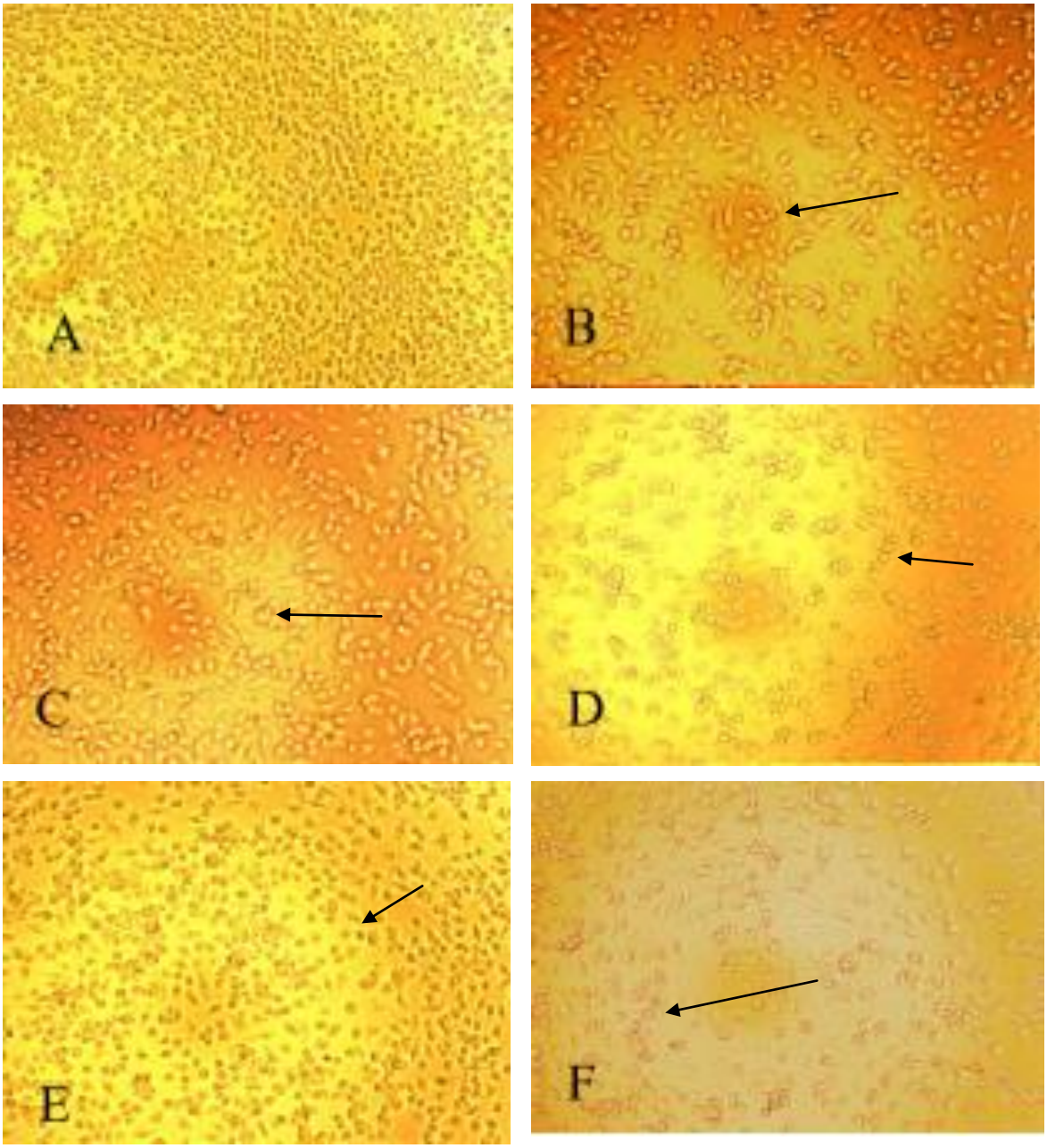


Figure 6. *Different pictures of LPS stimulated macrophages. Black arrows indicate stimulated macrophages after 24 hours. Panel "A" not induced by LPS. "B" induced by LPS concentration of 1 µg/mL. "C" induced by LPS concentration of 2 µg/mL. D" induced by LPS concentration of 5 µg/mL. "E" induced by LPS concentration of 10 µg/mL."F" induced by LPS concentration of 20 µg/mL.*

3.4 **Trophoblast NO production after 8 hours stimulation by lipopolysaccharide**

For each lipopolysaccharide (LPS) concentration, four different absorbance values were collected (n = 1). For example, the sample with LPS concentration of 1 μg/mL was put in four different cuvettes and these cuvettes were put in spectrophotometer to measure four different absorbance values. The mean and standard deviation (SD) was calculated for each.

Table 7.

Mean *NO_2^- absorbance values for trophoblast after 8 hours.*

LPS concentration (μg/mL)	0	1	2	5	10	20
Average NO_2^- Absorbance	-0.0034	-0.0030	-0.0038	-0.0051	-0.0042	-0.0038
SD (s) $\pm$	0.0009	0.0006	0.0009	0.0005	0.0005	0.0005

Note. LPS = Lipopolysaccharide, NO_2^- = Nitrite, SD = Standard deviation

No further calculation was carried out because of the negative values of trophoblast absorbance. These negative values are difficult to explain. The negative (close to zero) values are most probably explained by a total lack of nitrite production.

3.5 **Trophoblast NO production after 24 hours stimulation by lipopolysaccharide**

(Figure 7)

For each lipopolysaccharide (LPS) concentration, four different absorbance values were collected, in similar manner as for NO production in trophoblasts after 8 hours (n = 1). The mean and standard deviation (SD) was calculated for each.

Table 8

Mean *NO_2^- absorbance values for trophoblast after 24 hours.*

LPS concentration (μg/mL)	0	1	2	5	10	20
Absorbance	0.0039	0.0039	0.0029	0.0026	0.0026	0.0035
SD (s)	0.0012	0.0009	0.0003	0.001	0.0009	0.0005

Note. LPS = Lipopolysaccharide, NO_2^- = Nitrite, SD = Standard deviation

No further calculation was carried out because of the very small values of trophoblast absorbance. The very small (close to zero) values are most probably explained by a total lack of nitrite production.

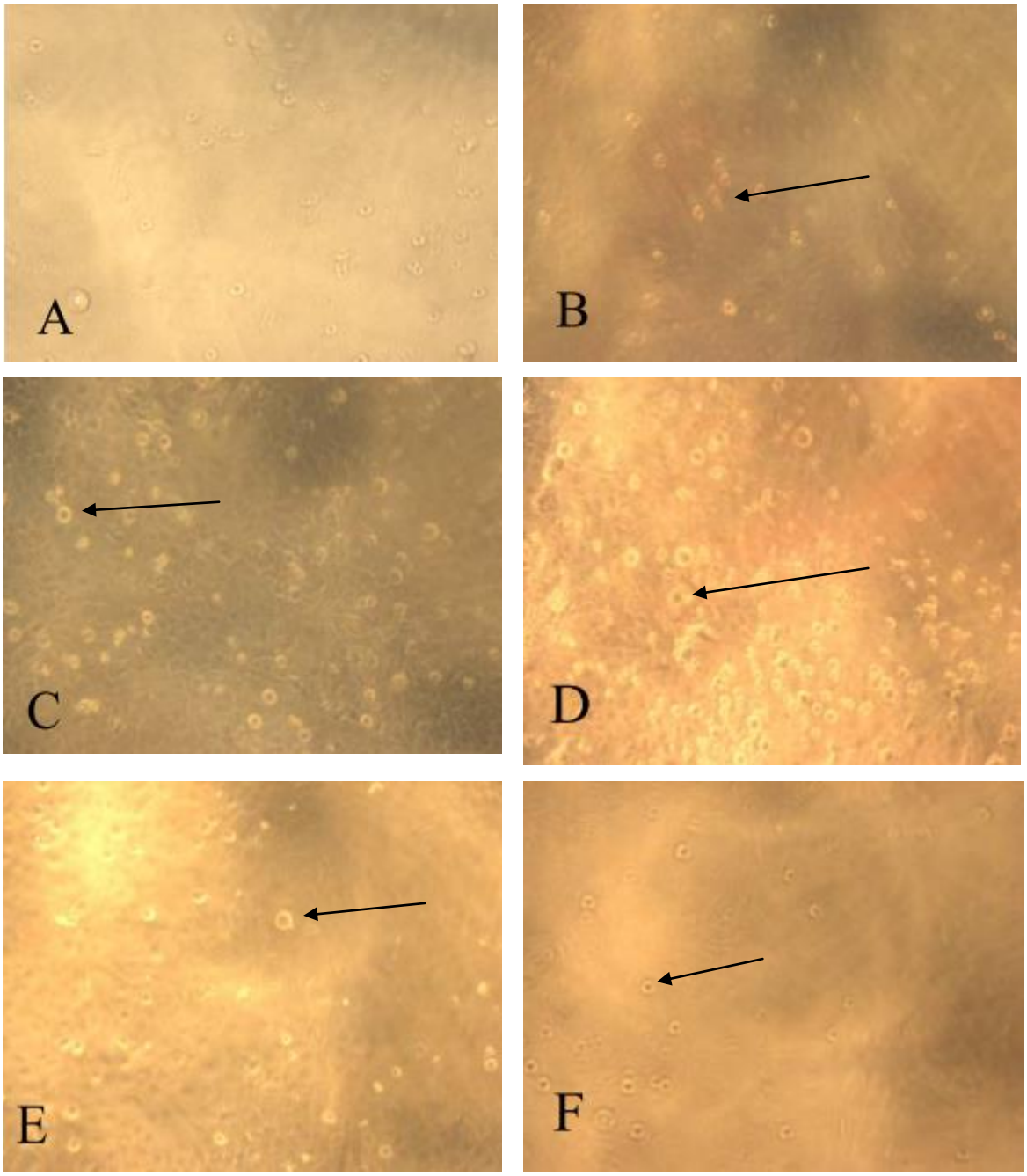


Figure 7. *Different pictures of LPS stimulated trophoblast. Black arrows indicate stimulated trophoblast after 24 hours. Panel "A" not induced by LPS. "B" induced by LPS concentration of 1 µg/mL. "C" induced by LPS concentration of 2 µg/mL. D" induced by LPS concentration of 5 µg/mL. "E" induced by LPS concentration of 10 µg/mL."F" induced by LPS concentration of 20 µg/mL.*

4.0 **Discussion**

Both trophoblasts and macrophages play an active role in the implantation process. Trophoblasts are the most important cells in the process of implantation. As mentioned earlier, nitric oxide produced by trophoblast may play a role in successful outcome of the implantation by dilating uteroplacental arteries[10], while nitric oxide produced by macrophage play role in regulation and apoptosis of trophoblast. [8]

When we compare the values of NO_2^- absorbance for macrophages after 8 hours (table 5) with the values of NO_2^- absorbance for macrophages after 24 hours (table 6), we notice that the biggest increase in value, of 900% occurred at LPS concentration of 2 μg/mL. The smallest increase in value for NO_2^- absorbance occurred at LPS concentration 1 μg/mL; 810%. This LPS induced increase in nitric oxide production in macrophages was both expected and in keeping with similar studies carried out by Cato[15] and Hirvonen[16].

The nitrite absorbance values for trophoblast did turn positive after 24 hours, but still the values were very small. If we take into consideration the NO_2^- absorbance value of control (no LPS stimulation (0 μg/mL concentration)), then, there is no indication of NO produced by the trophoblast, induced by any concentration of LPS. By comparing the NO_2^- absorbance values for trophoblast after 8 hours (table 7) with the NO_2^- absorbance values for trophoblast after 24 hours (table 8), we can notice that the NO_2^- absorbance values at LPS concentration value of 5 μg/mL changed from -0.0051 to 0.003, remains nearly zero. This lack of nitric oxide production by trophoblast correlates with previous studies. Based on the other relevant studies, we can conclude that lack of nitric oxide production in trophoblast may be because of LPS concentration was too low or time limit for measurement was too short. Lyall et al. used Northern analysis method to show that mRNA for eNOS and iNOS in trohpblast was undetectable after 24 hours. [17] Asagiri *et al.* in their studies confirmed significant amount of nitrite demonstrated by trophoblast with 100 μg/mL concentrations of LPS at day1. [18]

The question we asked ourselves at the start of the experiment was whether en excess of macrophage NO over trophoblast NO is responsible for failure of implantation by causing apoptosis of trophoblast if both cells are present at the same place, at same time and under similar conditions. Our results suggest that NO produced by the macrophages is greater than the NO produced by trophoblasts under similar conditions. Kaufman has shown in his studies that activated macrophages producing NO can activate other macrophages and lead to a

vicious cycle. [10] Many activated macrophages producing NO may lead to excessive apoptosis of trophoblast and may lead to failure of the implantation process. However, the two cell types do not always coexist in the same place, according to Kaufman: ["In normal pregnancy, the walls of uteroplacental arteries are largely devoid of macrophages and become invaded by the trophoblast."][10]

In our study, we used non-human cells like Murine macrophage-like cell line, J744.1 and Trophoblast (HTR-8/SVneo) cell line. This was because of restrictions imposed in the use of human cells and ethical aspects of such a study. It is possible that human cells may behave in similar fashion or not. Human cells were used in some other studies with similar results like Lyall used human trophoblast to show that mRNA for eNOS and iNOS was not detectable after 24 hours. [17] We used nitrite absorbance method, while in other, more advanced studies sophisticated methods were used like Northern analysis method, DNA sequencing or RT-PCR for detection of mRNA for eNOS or iNOS, and they reached similar conclusions.

Another possible drawback with our study was that only one successful experiment was conducted with each type. That means only one experiment was conducted with Sodium Nitrate solutions (n=1), only one successful experiment was conducted with macrophages (n =1), and only one successful experiment was conducted with trophoblast (n =1). We were able to grow and stimulate macrophages only once, out of three attempts. Half of these macrophages were used after 8 hours another half was used after 24 hours. Similar method was used for trophoblast. Despite the fact that we conducted only one experiment of each, we got the results that were supported by other studies, as mentioned above. Caution is therefore needed in interpreting our findings, which should ideally be reproduced in a larger number of samples.

5.0 **Conclusion**

At the fetomaternal interface both macrophages and trophoblasts are involved in the implantation process, both produce nitric oxide, and both may or may not be at the same place or under similar environment. Nitric oxide produced by the two may be quantitatively different, and serve different purposes. Our study provides modest evidence to suggest that if both these cells were to be exposed to similar inflammatory conditions, for equal amount of time, the quantitative values of nitric oxide produced by these two are very different, with potential significance for the balance between successful trophoblastic invasion and macrophage mediated apoptosis.

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