EXPRESSION OF iNOS MESSAGE IN HUMAN BUCCAL MUCOSAL (KERATINOCYTES) CELL LINE TR146 USING RT-PCR AND IMMUNOCYTOCHEMISTRY

[1]MUHAMMAD RIZWAN, BDS, MSc
[2] SADIA RIZWAN, BDS, M Phil (Trainee)

ABSTRACT

This study was carried out at the department of Oral Pathology, Queen Mary College of Medicine and Dentistry, Barts and The London to determine whether human oral buccal mucosal (keratinocytes) cell line, TR 146, expressed iNOS message and whether the expression of iNOS is varied when TR146 cells were exposed to different cytokines such as IL-15, IL-8, IL-1 β, or TNF- α using RT-PCR and Immuno-cytochemistry and to determine the effect of the above mentioned cytokines on NO function by measuring nitrite production in TR146 cells. Immuno-cytochemistry analysis revealed that TR146 cells expressed iNOS proteins when incubated with IL-15 and IL-8 and a modest increase was seen with IL-1 β / TNF-α. RT-PCR for iNOS indicated a marked increase in expression when the cells were exposed to IL-8, IL-15 or IL-1β/ TNF-α. NOS activity was assessed by measuring nitrite activity. It was observed that treating the cells with cytokines caused significant increase in nitrite levels, except in the case of IL-8. These results suggest that TR146 cells expressed iNOS , the levels of which varied with various cytokines. It is clear that IL-15, IL-1β / TNF-a and IL-8 are regulators of iNOS expression in oral keratinocytes and affect NOS activity.

Key words: *NOS, LPS, Cytokines, TR146, Immunocytochemistry, RT-PCR. Keratinocytes*

INTRODUCTION

Nitric oxide (NO) is a dissolved gas, produced from the amino acid L-arginine by NO synthase (NOS) and is thought to have a physiological and patho-physiological roles in maintaining blood pressure. NO causes smooth muscle cells in the vessel walls to relax, so that the vessels dilate and blood flows more freely through them.[1] Lipooxygenase products regulate NO production in IL-1â stimulated vascular smooth muscle cells (VSMCs).[2]

NO is also used by many nerve cells to signal neighboring cells. Dissolved NO diffuses readily out of the cells that makes it and into neighboring cells.[1] NO stimulates soluble guanylyl cyclase (sgc) to produce cGMP in endothelial cells.[3] NO prevents 6-hydroxydopamine-induced apoptosis in PC12 cells through cGMP-dependent p13 kinase/ AKT activation.[4]

Endothelial cells, the flattened cells that line every blood vessel, release NO in response to stimulation by nerve endings, and this NO causes smooth muscle cells in vessel walls to relax. Dendritic cells and macrophages produce NO in response to LPS stimulation.[5] NO is also produced by intestinal epithelial cells in response to challenge from gut microorganisms and may be involved in host defense against inner pathogens.[6]

NO is important in an early stage of the innate immune response.[5] NO is shown to have physiological and pathophysiological roles in neurotransmission, blood pressure homeostasis, and immunological responses.[7]

Nitric oxide synthase is an enzyme that produces nitric oxide from L-arginine. Three isoforms of NOS have been identified and cloned. Endothelial NOS is expressed in endothelial cells, neural NOS is expressed constitutively in the neural cells and inducible nitric oxide synthase (iNOS) is seen a variety of cells types.[6,8,9,10] Nitric oxide synthase governs the production of NO during nervous system development as well as in certain disease states such as stroke, multiple sclerosis Parkinson's disease, and HIV dementia.[11]

[1] Assistant Professor, Oral Pathology, Faculty of Dentistry, University of Lahore Dental College, 1-Km Raiwind Road, Lahore. Cell: 0333-5435866/0331-5330326, Tel: 0423-35504256, e-mail: dr_riz2000@hotmail.com

[2] M Phil (Trainee)

iNOS is an isoform of NOS and its expression is seen in a wide variety of cells such as macrophages, hepatocytes, and keratinocytes and is induced by several agents as LPS and cytokines and has the ability to synthesize large amounts of NO.[6, 8,9,10]

iNOS expression has also been observed in the basal layers of gingival epithelia of non-inflammaed and inflamed periodontal tissues.[12] Pulmonary epithelial cells express iNOS [6] as do human intestinal epithelial cells.[13] Lipooxygenase products regulate iNOS production in IL-1â stimulated vascular smooth muscle cells (VSMCs).[2] Agents such as LPS and cytokines induced expression of iNOS.[6, 8,9,10] Recent evidence suggests the possible involvement of iNOS in the development and maintainence of hypertension in certain animal models.[1]

PKC may be involved in the post-translational modification of iNOS and in the regulation of the availability of iNOS substrates.[14] Studies suggest that the MAP kinase pathway is partly involved in cytokine-induced iNOS expression in rat vascular smooth muscle cells (VSMCs).[15]

Cytokine or LPS-induced inos is involved in the abrogation of intracellular pathogens and inflammatory responses.[13, 16, 17]

METHODOLOGY

Present study was carried out at The department of Oral Pathology, Queen Mary College of Medicine and Dentistry, Barts and The London, UK, The human oral keratinocytes cell line, TR146 was obtained from Dr Alan Cruchley, Deptt of Oral Pathology. Cell line was cultured in T75 cm2 plastic tissue culture flasks containing 10ml of Dulbecco's Modified Eagle growth Medium (DMEM), as growth medium, supplemented with 10% fetal bovine serum (FBS) and 0.1% penicillin/streptomycin (PS). Cells were maintained in a humidified atmosphere of 95% air / 5% CO2.

The experiments of this project were carried out in three phases. Firstly, Immunocytochemistry was performed to determine iNOS expression in TR146 cells. Secondly, Reverse transcription followed by polymerase chain reaction (RT-PCR) was carried out to determine whether the cells expressed iNOS mRNA. Thirdly, nitrite levels in the cells were measured in response to various cytokines to determine if iNOS is active in TR146 cells.

Immunocytochemistry

It was carried out to determine whether human oral keratinocytes express iNOS message. Oral keratinocytes (TR146) cells were cultured in a growth medium, Dulbecco's Modified Eagle Medium (DMEM by invitrogen, Paisley, Scotland), supplemented with 10% fetal Bovine Serum (FBS) and 0.1% Penicillin/ Streptomycin (PS). Cells were maintained in a humidified atmosphere of 95% air / 5% CO2. After growth, cells from the flasks cultured in DMEM growth medium were obtained and separated by separating solution. Immunocytochemistry was performed in 24-wells plate using, Anti-human IL-15R-α antibody (R& D Systems, Human IL-15R- α extra-cellular domain specific goat IgG type) and Sf 21-derived rhIL-15R-α extra-cellular domain Immunogen) (Table 1). The experiment was repeated 3 times.

TABLE 1: TREATMENTS USED FOR VARIOUS CYTOKINES IN 24 WELLS PLATE

Treatment Materials	Control	IL-15	IL-1 β / TNF- α	IL-8
PBSµl	200µl	200µl	200µl	200µl
iNOSµl	200µl	200µl	200µl	200µl

2- RT-PCR (Reverse Transcription-Polymerase Chain Reaction)

PCR is a technique for the in-vitro amplification of specific DNA sequences by the simultaneous primer extension of complimentary strands of DNA. In RT-PCR, single stranded RNA is used as a technique to template to synthesize double stranded DNA. The purpose of this experiment was to determine whether oral epithelial cells express mRNA encoding the gene for iNOS.

Oral keratinocytes were cultured in a growth medium DMEM. Cells were separated for PCR and following steps were followed

RNA extraction

Extraction of RNA from the cells. The concentration of the RNA was measured by using UV-VIS Spectrophotometer (UV-mini 1240 Spectrophotometr by Shamzadu Deutschland, GmbH) at A260 and A280 nm. And RNA concentration was calculated using the following formula:

A260 x dilution factor = µg RNA / ml

cDNA Synthesis

After RNA extraction, cDNA synthesis was performed using following materials and protocol (Table 2).

Dctp, Aatp, dgtp, dttp (100Mm), Promega ltd. UK.

DTT (0.1M), Oligo Dt Subscript. Primer (0.5µg / µl), RNase H (3U /µl), Superscript RT, 5 x 1st strand buffer all from Invitrogen.

RNase inhibitor (40U/ul), Helena Biosciences,

UK. RNase A (10mg/ml), Sigma, Poole, UK.

TABLE 2: MATERIALS USED FOR cDNA SYNTHESIS

Materials	Control	IL-15	IL-8	IL-1β/ TNF
Oligo Dt	1µl	1µl	1µl	1µl
RNA (2ug)	5µl	5µl	5µl	5µl
DEPC Water	4µl	4µl	4µl	4µl
Total volume	10µl	10µl	10µl	10µl

Polymerase Chain Reaction (PCR)

PCR reagents 10x PCR Buffer 5.0µl, Distilled water 42.8µl, dNTPs (200µM) 0.5µl, sensenprimer 0.2µl, antisense primer 0.2µl, Cdna 1.0µl, Taq polymerase 0.3µl and total of 50.0µl were set up in 0.5ml microfuge tubes and mixed gently.

DNA was substituted for water as a negative control for PCR. And then PCR reactions were put through a cycling program in a hybaid thermocycler. The program was run as mentioned in table 3.

Then about 10µl of the PCR products were mixed with 2µl of loading dye and ran in a 1% agarose gel and then viewed by UV illumination and pictures taken.

Nitrite Assay

The accumulation levels of nitrate and nitrite resulting from NO produced by IL-15 stimulated human buccal epithelial cells were measured, as NO is rapidly converted into these two stable end products.[18]

The accumulation level of nitrite resulting from NO activity by IL-15 stimulated TR146 cells, was measured using as in-house 96 well assay. These assays were performed by the help of Dr Justin Doel, Department of Oral Microbiology, Queen Mary College Of Medicine and Dentistry, London, UK.

Cells cultured in growth medium, were removed from the culture substrate by treatment with trypsin.

TABLE 3

Process	Temperature	Time	Cycles
DNA denaturation	94 c	5 minutes	1 cycle
DNA denaturation	94 c	1 minute	35 cycles
Primer melting/ annealing	60c	I minute	35 cycles
Primer extension	72c	2 minutes	35 cycles
Primer extension	72c	7 minutes	1 cycle

Cells were stimulated with by IL-15 100ng/ml, IL-8 100ng/ml, or IL-1 β /TNF-α 100ng/ml overnight and were incubated (in a 500µl volume) overnight. Medium was then harvested and stored at-20 degree C until assayed for nitrite. Nitrite concentrations were determined in 96-well plates, in duplicate using 50µl sample volume of each treatment used. Each sample was treated with 50µl sulphanilamide solution and incubated for 5 minutes at room temperature protecting from light. Then 50µl NED sol. (N-1-naphthylethylenediamine dihydrochloride in water) was added and incubated for 5 minutes at room temp. protecting from light.

Plates were then read using a plate reader at 540nm (Table 4). Values of nitrite were estimated by comparing with a standard curve of nitrite concentrations including 250µm, 125µm, 62.2 µm, 31.1µm, and 0µm nitrite (Fig 3).

RESULTS

Fig.1 : Immunostaining showing the presence of iNOS in TR164 cells. A, no primary antibody; B, no treatment; C, IL-15 (100ng/ml); D, IL-8 (100ng/ml); E, IL-1 β/TNF-α (100ng/ml). Antibody was used at 1:100. Cells were fixed and stained. Experiments were carried out thrice and magnification used was 200x

Immunostaining of the treated and untreated cells was carried out to see the expression of iNOS protein in TR146 cells. Figure 1 showed that cells with no primary antibody and cells with no treatment showed no expression of iNOS. (Fig 1A and 1B). Cells treated

TABLE 4: NITRITE ASSAY

Nitrite (µm)	At 540 nm
0	0.04
31.1	0.45
62.2	1.1
125	2.3
250	4.1

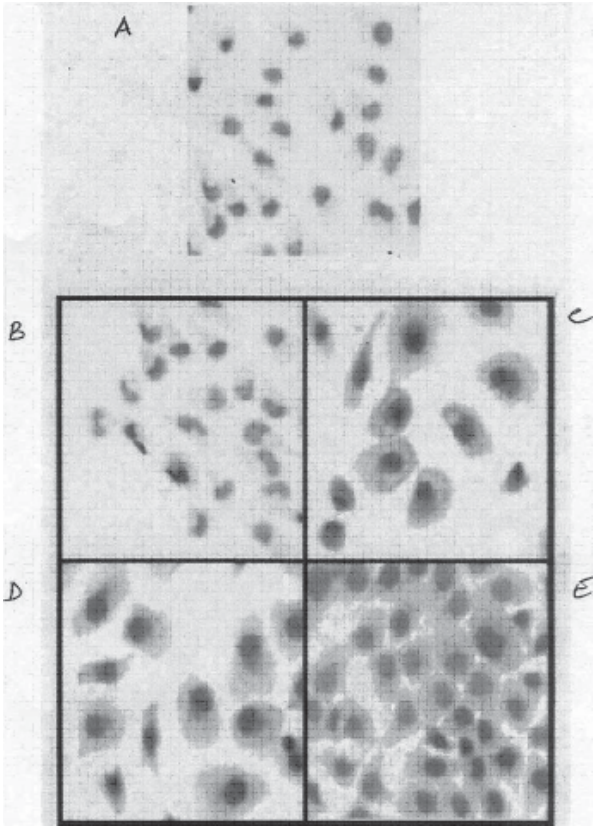

Fig 1: Immunostaining to see the expression of iNOS in TR146 cells

with IL-15 and IL-8 showed marked expression of iNOS. (Fig 1 C and 1 D) illustrates that the intense red color indicates increased expression of iNOS in these cells. Fig 1 E shows that cells treated with IL-1 β / TNF-α had a modest increase in iNOS protein expression.

Fig 2: PCR was carried out for iNOS using cDNA obtained from TR146 cells.

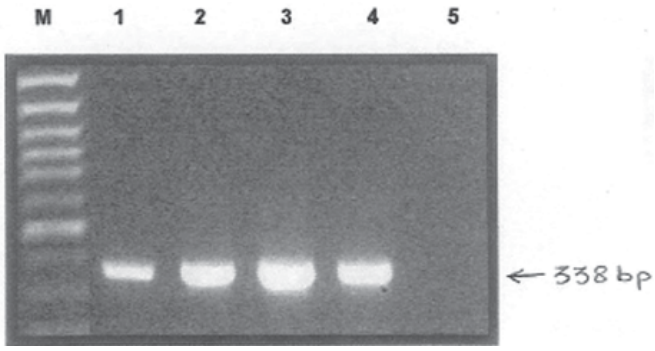

Fig 2: Reverse Transcription-Polymerase Chain Reaction (RT-PCR): Detection of iNOS mRNA expression by RT-PCR

M, DNA ladder; 1, untreated cell; 2, IL-15 (100ng/ml) treated cell; 3, IL-8 (100ng/ml) treated cells; 4, IL-1 β/ TNF-α (100ng/ml) treated cells; 5, Water. The size of the amplified product is indicated by the bp and arrow. Experiments were carried out 3 times.

RT-PCR to see the expression of iNOS mRNA was carried out. From the results obtained, it can be seen that the cells with different treatments showed a marked expression of iNOS mRNA compared to untreated cells. The cells which were treated with IL-8 seem to show more iNOS mRNA expression than the cells treated with IL-15 and IL-1 β / TNF-α.

Fig 4: Graph illustrating the effect of different cytokine treatments on nitrite production in TR146 cells. Cells were treated overnight with IL-15 (100ng/ml), IL-8, IL-1 β /TNF-α and the medium assayed for nitrite. Bars indicate the mean and standard deviation of 3 experiments. Statistical significance calculated by student's t-test. *, $p<0.01$; ***, $p<0.001$ compared to control.

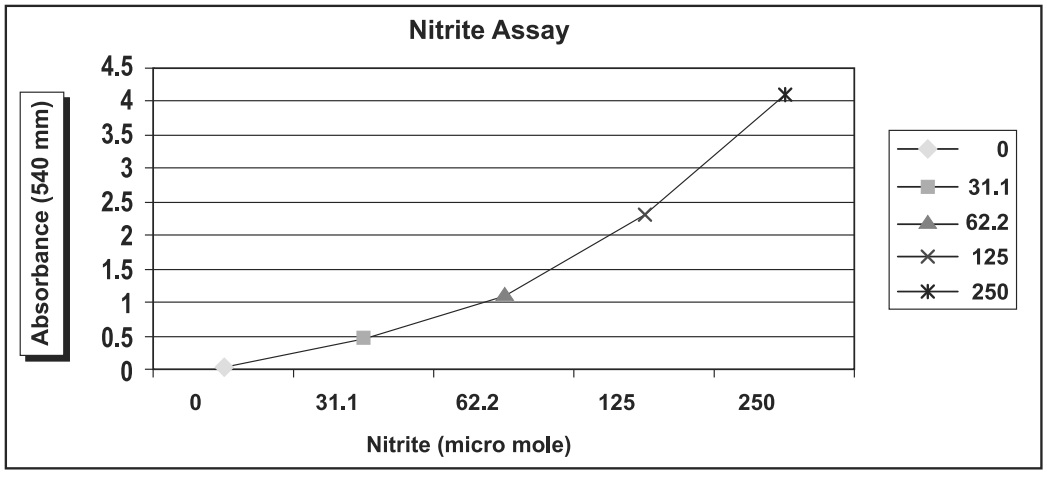

Fig 3: This is a typical standard curve for the nitrite assay. This graph shows that the concentration of nitrite (μM) increases with increases in absorbance reading.

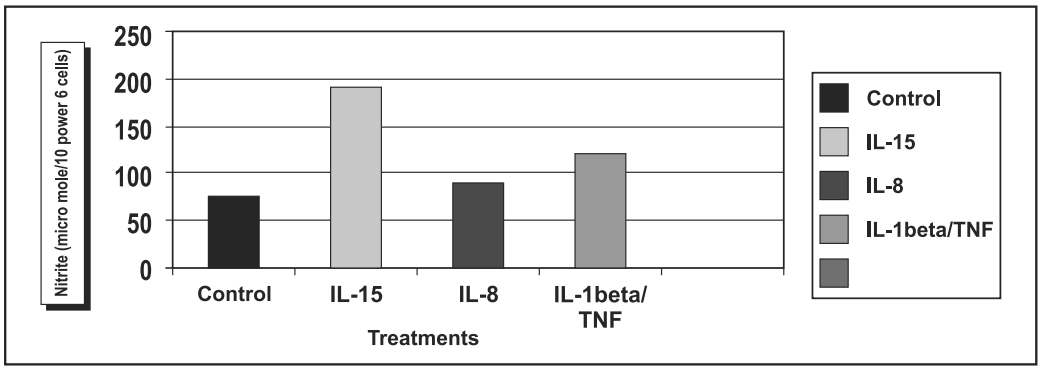

Fig. 4: Detection of nitrite production by TR146 cells on stimulation with different treatment

The effect of IL-15, IL-8, and IL-1 β /TNF-gmma on NO activity in TR146 cells was estimated by measuring nitrite levels. TR146 cells were incubated with or without 100ng/ml of cytokines. Nitrite levels in the culture supernatants were determined. Untreated cells demonstrated some nitrite production where as IL-15 clearly showed increases (more than double the control) production of nitrite. IL8 and IL-1β /TNF- α also showed increased production of nitrite than the controlled cells. Data indicates that these cells have NOS activity which increased when treated with various cytokines.

DISCUSSION

Prior to the present study, it had been reported that IL-15 protein was expressed in a constitutive manner in basal epithelial keratinocytes in healthy human gingivae, and that IL-15 and IL15-R α chain mRNA could be detected in human gingival epithelial cells (HGEC) by RT-PCR analysis.

Previous studies have reported higher levels of iNOS mRNA expression in the periotoneal macrophages in IL-15 transgenic mice after stimulation of Mycobact-erium bovis bacillus, compared with those of wild mice.[19] Another study showed both iNOS and IL-15R mRNA expression were up-regulated in mouse dendritic cells after stimulation of LPS or propionebacterium acnes.[20] These studies suggested that IL-15 may be involved in iNOS expression in other cell types. This study directly demonstrated that human oral keratinocytes cells express IL-15R and IL-15 has the ability to induce iNOS expression and nitrite production.

Studies have also found that gingival keratinocytes can be induced to express iNOS by stimulation with IL-1β, IL-8, TNF-α, or LPS as well as their combinations.[6,8,22] It has been reported that epithelial cells highly express iNOS at both mRNA and protein levels in inflammatory lesions, including those of Periodontitis.[12] Although the physiological and pathophysiological roles of iNOS expression in periodontal lesion are not yet fully defined, it is expected that hyperproduction of NO in inflamed periodontal lesions may be involved in epithelial barrier dysfunction and alveolar bone resorption. IL-15 receptors are known to be expressed in a variety of tissues, including epithelial cells [25], β cells and NK cells.[24]

It was shown that IL-15 was also able to induce iNOS and NO production by epithelial cells.[13,23,26] Our present findings provide new evidence that an oral keratinocytes cell line express IL-15R and iNOS and that IL-15 is also a potent regulatory cytokine for the stimulation of iNOS and nitrite production in these cells. Immunostaining to see the presence of iNOS in TR146 cells (Fig 4) show that cells treated with IL-15 and other inflammatory cytokines IL-8, and IL-1β/ TNF-α caused an increase in IL-15R and iNOS expression in these cells.

IL-15 acts via IL-15R, which shares β and Gamma subunit with IL-2R. however, unlike IL-2R, IL-15R expression is widely distributed throughout a variety of tissues and cells. It remains uncertain what kind of intracellular signal transduction is involved in IL-15R mediated iNOS expression in TR146, though several signaling pathways have been implicated in the up-regulation of iNOS mRNA in HGEC. Among these factors, NF-kappaB is a pivotal transcription inducer of iNOS expression in various cells.

It is possible that endothelial cells and fibroblasts underlying buccal epithelial cells are also influenced by IL-15 generated from these cells. A recent report has revealed that stimulation with IL-15 increases hyaluronan synthesis by endothelial cells,[27] and gingival fibroblasts. Since hyaluronan is known to function as a scavenger of nitric oxide (NO) derivatives

such as peroxynitrite,[21] which exerts deleterious effects on host cells, the IL-15 induced hyaluronan generation by endothelial cells and fibroblasts may contribute to the prevention of NO associated tissue damage.

In summary, findings of the present study demonstrate that TR146 cells express IL-15R and iNOS message, and IL-15 is a potent inducer and regulator of iNOS expression and nitrite production in these cells.

REFERENCES

1 Alberts B, Bray D, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Essential cell biology, an introduction to the molecular biology of the cell. Garland Publishing Inc., USA. 1997; 44-49

2 Hashimoto T, Kihara M, Yokoyama K, Fujita T, Kobayashi S, Matsushita K, Tamura K, Hirawa N, Toyo Y, Umemura S. Lipooxygenase products regulate nitric oxide and inducible nitric oxide synthase production in interleukin-1beta stimulated vascular smooth muscle cells. Hyperten. Res. 2003; 26: 177-84

3 Rivero-vilches FJ, De Frutos S, Saura M, Rodriguez-puyol D, Rodriguez-Puyol M. Differential relaxing responses to particulate or soluble Guanylyl Cyclase activation on endothelial cells. A mechanism dependent on PKG-1alfa activation by NO/cGMP. Am J physiol Cell Physiol. 2003; 285: C891-98

4 Ha KS, Kim KM, Kwon YG, Bai SK, Nam WD, Yoo YM, Kim PK, Chung HT, Billiar TR, Kim YM. Nitric oxide prevents 6-hydroxydopamine-induced apoptosis in PC12 cells through cGMP-dependent P13 Kinase /AKT activation. FASEB J. 2003; 17: 1036-47

5 Ohteki T, Suzue K, Maki C, Ota T, and Koyasu H. Critical role of IL-15—IL-15R for antigen-presenting cell functions in the innate immune response, Nature Immunology. 2001; 2: 1138-43

6 Hoffmann G, Grote J, Friedrich F, Mutz N, Schobersberger W. The pulmonary epithelial cell line L2 as a new model for an inducible nitric oxide synthase expressing distal air way epithelial cell, Biochem. Biophys. Res. Commun. 1995; 217: 575-83

7 Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N. Engl. J. Med. 1993; 329: 2002-12

8 Asano K, Chee CB, Gaston B, Lilly CM, Gerard C, Drazen JM, Stamler JS. Constitutive and inducible nitric oxide synthase gene expression, regulation and activity in human lung epithelial cells. Proc Natl Acad Sci USA. 1994; 91: 10089-93

9 Eckmann L, Laurent F, Langford TD, Hetsko ML, Smith JR, Kagnoff MF, Gillin FD. Nitric oxide production by human intestinal epithelial cells and competition for arginine as potential determinants of host defense against the lumen-dwelling pathogen giardia lamblia. J. Immunol. 2000; 164: 1478-87

10 Fried M, Prives C, Botchen M, Grodzicker T, Sharp P (Eds). Cancer cells: DNA tumor viruses, Cold spring harbor laboratory, Cold spring harbor, New York. 1986; 4: 1-16

11 Lee J, Ryu H, Ferrante RJ, Morris SM Jr, Rattan RR. Ttanslation control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. Proc. Natl. Acad. Sci USA. 2003; 100: 4843-48

12 Kendall HK, Haase HR, Xiao Y, Barthold MP. Nitric oxide synthase type-2 is synthesized by human gingival tissues and cultured human gingival fibroblasts. J Perio. Res. 2000; 35: 194-200

13 Salzman AL, Eaves Pyles T, Linn SC, Denenberg AG, Szabo C. Bacterial induction of inducible nitric oxide synthase in cultured human intestinal epithelial cells. Gastroenterol. 1998; 114: 93-102

14 Han YL, Kang J, Li AH,. Protein kinase c and protein tyrosine kinase mediate lipopolysaccharide- and cytokine-induced nitric oxide formation in vascular smooth muscle cells of rats. Sheng Li Xue Bao. 2003; 55: 265-72

15 Doi M, Shichiri M, Katsuyama K, Marumo F, Hirate Y. Cytokine-activated p42/p44 MAP kinase is involved in inducible nitric oxide synthase gene expression independent from NK-Kappa B activation in vascular smooth muscle cells. Hypertens Res. 2000; 23: 659-67

16 Evas Pyles T, Murthy K, Liaudet L, Virag L, Ross G, Soriano FG, Szabo C, Salzman AL. Flagellin, a novel mediator of salmonella-induced epithelial activation and systemic inflammation: 1kB alfa degradation, induction of nitric oxide synthase, induction of inflammatory mediators, and cardiovascular dysfunction. J Immunol. 2001; 166: 1248-60

17 Oswald IP, Eltoum I, Wynn TA, Schwartz B, Caspar P, Paulin D, Sher A, James SL. Endothelial cells are activated by cytokine treatment to kill an intravascular parasite, Schistosoma Mansoni, through the production of nitric oxide. Proc. Natl. Acad. Sci USA. 1994; 91: 999-1003

18 Moncada S, Palmer RM, Higgs EA. Nitric oxide: Physiol. Pathophysiol. And Pharmacol, Pharmacol Rev. 1991; 43: 109-42

19 Umemura M, Nishimura H, Hirose K, Matsuguchi T, Yoshikai Y. Over expression of IL-15 in vivo enhances protection against mycobacterium bovis bacillus Calmette-Guerin infection via augmentation of NK and T cytotoxic I responses. J. Immunol. 2001; 167: 946-56

20 MacDonald AS, Straw AD, Bauman B, Pearce EJ. CD8 dendritic cell activation status plays as integral role in influencing Th2 response development. J. Immunol. 2001;167: 1982-88

21 Li M, Rosenfeld L, Vilar RE, Cowman ML. Degradation of hyaluronan by peroxynitrite. Arch. Biochem. Biophys. 1997; 341: 245-50

22 Gerharz DB, Fehsel K, Suschek C, Michel G, Ruzicka T, Bachofen VK. A proinflammatory activity of interleukin 8 in human skin: expression of the inducible nitric oxide synthase in psoriatic lesions and cultured keratinocytes. J. Exp Med. 1996; 184: 2007-12

23 Kwon S, George SC. Synergistic cytokine-induced nitric oxide production in human alveolar epithelial cells. Nitric oxide 1999; 3: 348-57

24 Tagaya Y, Bamford RN, Defilippis AP, Waldmann TA. IL-15, a pleiotropic cytokine with diverse receptor/ signaling pathways whose expression is controlled at multiple levels. Immunity 1996; 4: 329-36

25 Giri JG, Kumaki S, Ahdieth M, Friend DJ, Loomis A, Shanebeck K, DuBose R, Cosman D, Park LS, Anderson DM. Identification and cloning of a novel IL-15 binding protein that is structurally related to the alfa chain of the IL-2 receptor. EMBO J. 1995; 14: 3654-63

26 Arany I, Brysk H, Tyring SK. Regulation of inducible nitric oxide synthase mRNA levels by differentiation and cytokines in human keratinocytes. Biochem. Biophys. Res. Commun 1996; 220: 618-22

27 Estess P, Nandi A, Muhamadzadeh M, Siegelman MH. Interleukin 15 induces endothelial hyaluronan expression in vitro and promotes activated T cell extravasation through a CD44 dependent pathway in vivo. J. Exp. Med. 1999; 190: 9-19