Semiquantitative RT-PCR analysis to assess the expression levels of iNOS in chicken macrophages

Nikbakht Brujeni,Gh.[1]*, Masood Teshfam, M.[2], Hossein Hassanpour,H.[2]

[1]*Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran- Iran.*

[2]*Department of Physiology, Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Tehran, Tehran- Iran.*

Abstract: **iNOS is inducible by a variety of factors related to inflammation and referred to as inducible NOS(iNOS). It is regulated at the level of gene expression; once expressed, it produces NO at a high rate. iNOS gene-expression profiling is an important tool in understanding molecular markers of the responses of cells and tissues to external factors. In this article a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) protocol was optimized to extract RNA (ribonucleic acid) from chicken spleen and to measure the expression levels of iNOS mRNAs from each sample. Detailed procedure was described for the analysis of iNOS levels. β-actin was used as an internal control to normalize for sample to sample variations in total RNA amounts and for reaction efficiency. Co-amplification of the iNOS gene with housekeeping gene (β-actin) provides a quantitative result. Changes in gene expression level may be monitored, while avoiding sample-to-sample loading variation.**

Introduction

One of the products of the oxidation of L-arginine in many organisms is nitric oxide (NO) that performs many diverse and significant biological functions (Arthur *et al*., 1996; Bredt and Snyder, 1994; Hampl *et al*., 2000; Kroncke *et al*., 2001). In the immune system, NO is synthesized by activated macrophages and acts as a cytotoxic, antiviral and tumoricidal agent and has been proved to be involved in non-specific defense mechanisms (Coleman, 2001; Kroncke *et al*., 2001; Liew, 1995; Liew and Cox, 1991; Van, 2001). NO also mediates important functions in other tissues and organs such as the lung, gastrointestinal tract, liver, pancreas, kidney, and the reproductive system (Arthur *et al*., 1996; Kobzik *et al*., 1993).

NO production involve the multi step reaction that is catalyzed by a single heme-containing enzyme, NO synthase (NOS), which exists in three isoforms: nNOS (neuronal NOS), a constitutive form which was initially identified in neurons; iNOS (inducible NOS), an inducible form from macrophages; eNOS (endothelial NOS), a constitutive form which was initially identified in endothelium (Bredt and Snyder, 1994; Griffith and Stuehr, 1995; Lamas *et al*., 1992; Nathan, 1992).

Among different NOS isoforms, iNOS is inducible by a variety of factors related to inflammation and therefore is often referred to as inducible NOS(iNOS)(Arthur *et al*., 1996; Coleman, 2001; Djeraba *et al*., 2002; Kroncke *et al*., 2001). It is regulated at the level of gene expression; once expressed, it produces NO at a high rate (Hampl *et al*., 2000). Due to the apparent correlation between iNOS gene expression and protein production, detection of mRNA (messenger ribonucleic acid) is an alternative method of demonstrating iNOS induction. Several techniques are currently available to measure

* **Corresponding author's email: nikbakht@ut.ac.ir, Tel: 021-, 61117057 Fax: 021-66427517**

changes in gene expression at the mRNA level. These include the northern blot, the RNase protection assay, insitu hybridization and the reverse transcription-polymerase chain reaction (RT-PCR)(Aust, 2003; Ausubel *et al*., 2000; DuBois *et al*., 1993; Gilliland *et al*., 1990; Melton *et al*., 1984; Reiner *et al*., 1994). RT-PCR is increasingly used to detect small changes in gene expression that would otherwise be undetectable (Freeman *et al*., 1999).

Although there has been considerable research on the biological functions of NO and the regulation of NOS in humans and rodents (Kobzik *et al*., 1993), little is known of NOS in non mammalian system such as chickens. The objectives of this study were to develop and validate a practical and sensitive RT-PCR system for quantitation of iNOS expression. This could help for better understanding the regulation of NOS at the molecular level in chickens.

Materials and Methods

Sample preparation and RNA Extraction from tissue: Chicken spleen was used as a source of macrophages. Spleen was removed aseptically. Single cell suspensions were prepared by gently teasing the organ on a steel sieve. Nucleated erythrocytes were eliminated by centrifugation at 400g. Total RNA was extracted by single step method of Ausubel *et al.,* (Ausubel *et al*., 2000). Briefly, 100mg of homogenized cells was prepared in 1 ml denaturing solution containing 4M guanidinium isothiocyanate. The homogenate was mixed sequentially with 0.1 ml of 2M sodium acetate pH 4, 1 ml water-saturated phenol, and 0.2 ml chloroform/isoamylalcohol (49:1). The resulting mixture was centrifuged, yielding an upper aqueous phase containing total RNA. Followed by 100% isopropanol precipitation, the RNA pellet was dissolved in 0.3 ml denaturing solution, reprecipitated with isopropanol, and washed with 75% ethanol. The RNA samples were resuspended in DEPC (diethylpyrocarbonate)- treated water and stored at -80°C. The yield and the integrity of each RNA sample were checked by spectrophotometrical measurement of 260A and agarose gel electrophoresis respectively.

Reverse Transcription: RNA was reverse transcribed to CDNA (complementary deoxyribonucleic acid) in a 20 µl final volume containing 1 µg of extracted RNA, 200ng random hexamer and 0.5 mM Deoxyribonucleotide triphosphate (dNTP) Mix. The mixture was heated to 65°C for 5 minutes, and added 4 U RNase inhibitor, RT buffer (50mM Tris-Hcl,75mM KCL,3mM MgCl$_2$), 10mM DTT and 200 unit M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). This mixture was incubated for 10 minutes at 25°C followed by 50 minutes incubation at 37°C. The prepared cDNA were heated at 75°C for 15 min to denature the MMLV-RT and then stored at -20°C.

Standard reaction for iNOS PCR: The PCR was performed in a total volume of 25µl. 2 µl of cDNA products were amplified with 1 unit of Taq polymerase (Promega, Germany) and 0.1mM of each dNTP in the buffer provided by the manufacturer which contains no MgCl$_2$, and in the presence of the specific primers for iNOS, together with the β-actin primers, used as an internal control. Primers were synthesized by Tib Molbiol (Germany). The following primers were used:

iNOS 5'- AGGCCAAACATCCTGGAGGTC -3'(Tm61,1^{o}C)
5'- TCATAGAGACGCTGCTGCCAG-3'(59,7^{o}C)
β-actin 5'-ACTGGATTTCGAGCAGGAGAT -3' (51°C)
5'- TTAGAAGCATTTGCGGTGGACAA -3' (53°C).

iNOS yielded an amplification product of 371 bp and β-actin of 468 bp. The melting temperatures (T_ms) indicated in brackets were reported according to primer data sheet provided by manufacturer (Tib Molbiol. Germany). Moreover, the PCR primers were selected in a way that the 5' and the 3' primers span different exons, so that the amplification product obtained from the cDNA were of different length from that obtained from contaminant genomic DNA comprising intronic sequences.

Reactions were carried out in the Ependorf PCR system 5331. PCR was performed with cycling program as follows: 1 cycle of 10 minutes at 94°C, 45 seconds at 65°C and 1 minute at 72°C; 27 cycles of 45 seconds at 94°C, 45 seconds at 65°C and 1 minute at 72°C. PCR products were in the exponential phase of

amplification. In this phase none of the RNAs analyzed reached a plateau at the end of the amplificated protocol. Moreover, in the selected conditions, two sets of primers used in each reaction did not compete with each other.

Each primer was used in a concentration range of 0.05 to 0.4 μM. Determination of the optimal concentration for each primer set was performed using different $MgCl_2$ concentrations in the standard of PCR reaction conditions and by adjusting the water volume consequently. We normally tested the following $MgCl_2$ concentrations: 1, 1.5, 2, 3 and 5 mM. The products were run on an agarose gel to choose the condition that gave the highest yield and specificity.

Appropriate number of cycles was determined by testing the different cycles of 15, 19, 23, 27, and 31 for both iNOS and β-actin amplifications.

Control reactions were performed to reveal whether the selected conditions are suitable for dual RT-PCR with both primers sets (iNOS and β-actin) at the same time. The same sample was amplified at the same time in the presence of the specific primers for iNOS, the internal control β-actin, and both sets together. Furthermore, different $MgCl_2$ concentrations for each reaction were tested. PCR products were then run on the same agarose gel for comparison and quantitation.

Certain condition, in that competition had not been detected, was used for determination of quantificated efficacy. Different concentrations of iNOS template were co-amplified with the same concentration of β-actin.

Acquisition of gel images and quantitative analysis: An aliquot of each PCR products were subjected to electrophoresis in 2% agarose gel and stained with 0.5 μg/ml ethidium bromide. Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a Sony XC-75 CE camera (Vilber Lourmat Inc. Cedex, France) and quantification of the bands was performed by Photo-Capt V.99 Image software (Vilber Lourmat Inc. Cedex, France). Relative densities were expressed as iNOS/β-Actin density.

Results

Concentration of 0.05-0.2 μM for primers usually is optimal. In this study iNOS with 0.1 μM of each of the two iNOS-specific primers and 0.05 μM of each β-actin-specific primers were optimized. Lower concentrations may limit the amplification and higher concentrations may cause non-specific amplification products.

The results of different cycling number have been demonstrated in Fig. 1. According to these results 27 cycles was selected for dual PCR of iNOS and β-actin.

Different concentrations of $MgCl_2$, in a range of 1-5 mM, were tested. As shown in Fig.2, iNOS worked best at 1, 1.5 and 2 mM $MgCl_2$, whereas the yield of β-actin did not change at the concentrations tested (Fig. 2). Any primer set behaves differently, and a narrower concentration range (for example 1, 1.5, 2, 2.5, 3 mM) was tested (data not shown). iNOS specific primers for the target RNA competed with the β-actin primers in 1 mM concentration of $MgCl_2$ (Fig. 3).

Fig. 4 shows an example of RT-PCR analysis performed in different concentration of iNOS template. According to the above results obtained from different conditions, 27 cycles and 2 mM $MgCl_2$ were selected for dual quantitative PCR. In this example co-amplification of 2.5 ng of β-actin with 0.125, 0.25, 0.5, 1 and 2ng of iNOS templates were illustrated. Quantification of the bands intensity expressed as iNOS/β-Actin relative densities (Fig. 4). β-actin did not change significantly and iNOS decreased under the serial dilution.

Discussion

Comparative gene-expression profiling is an important tool in understanding molecular markers of the responses of cells and tissues to external factors (Carding *et al*., 1992; Crawford *et al*., 2002; Djeraba *et al*., 2002; Gilliland *et al*., 1990; Han *et al*., 1999; Svetic *et al*., 1991). With increasing microarray data, molecular patterns are emerging but the acquisition of these data is expensive, difficult to customize and not well standardized. Once genome-wide scans identify differentially expressed genes in a given condition, cheaper, more easily customized methods will be needed for evaluating the expression of these
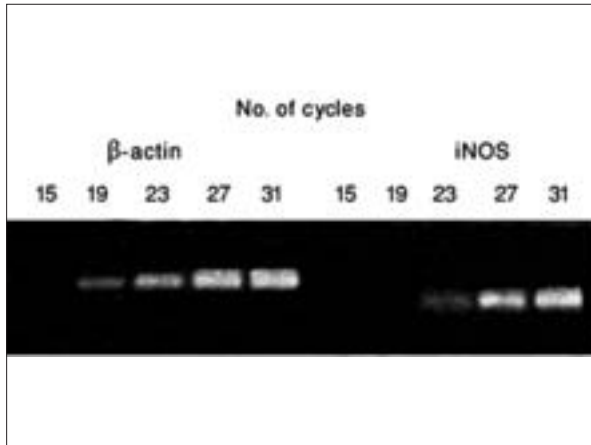
Fig. 1- Determination of the exponential range of amplification for iNOS and β-actin. The analysis was performed in 1 mM MgCl₂ at 65°C. The intensity of the iNOS amplification product increased up to 31 cycles, whereas β-actin reached a plateau at 27 cycles.
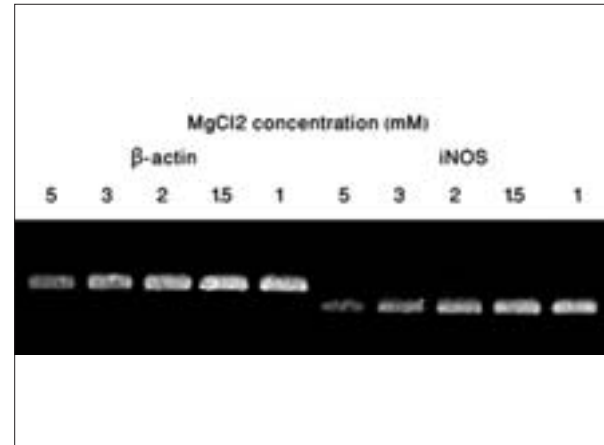

Fig. 2- Effect of different MgCl₂ concentrations in the PCR reaction. Analysis was performed on the spleen tissue. iNOS amplification was optimal at 1, 1.5 and 2 mM MgCl₂ and decreased gradually at higher concentrations. The intensity of the β-actin amplification product did not change significantly.

genes in large population samples.

Reverse transcription-polymerase chain reaction assays that attempt to quantify gene expression require internal controls (Ali *et al*., 1997, Carding *et al*., 1992; Freeman *et al*., 1999). Because of the independent nature of amplifying two different sequences, each with its own respective primer pair, housekeeping gene internal controls downgrade RT-PCR assays to a semi-quantitative level of accuracy. However, the simplistic approach of using such endogenous internal controls does offer some practical advantages to RT-PCR, when precise quantification is not required. Firstly, the ratio of target to control transcripts in a single RNA sample should always be consistent, thus the quantity of RNA added to reactions is not critical. Also, there is no need to spike samples with an equivalent amount of the control substance. Furthermore, housekeeping genes are versatile and may be used as controls for any number of different target genes. Lastly, to utilize housekeeping gene internal control all that is required is the primer set.

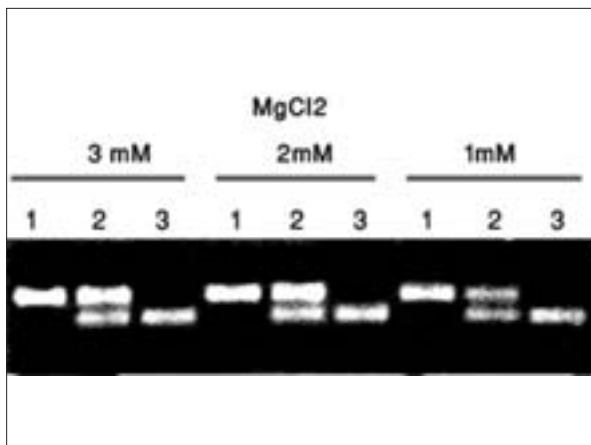Commonly used to quantitate mouse and human

Fig. 3- Control for competition between different primer sets. Reactions were performed in the same conditions for lanes 1, 2 and 3. Lane 1 contained the β-actin primer set only, lane 3 the Specific iNOS primers for the target RNA, and lane 2 both primer sets. In the selected conditions 2 and 3 mM MgCl₂, iNOS and β-actin did not compete. Specific primers for iNOS competed with the β-actin primers in 1 mM MgCl₂ concentration.
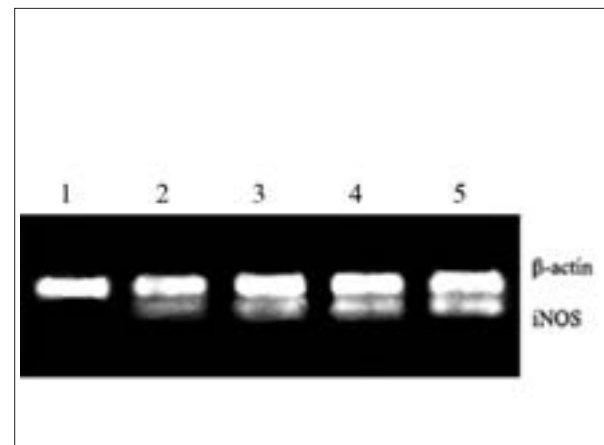

Fig. 4- Example of analyses performed on different concentration of target iNOS in the same concentration of β-actin . Lane 1-5 co amplification of 2.5 ng of β-actin with respectively 0.125, 0.25, 0.5, 1 and 2ng of iNOS templates. N/A indicates the relative level of the iNOS amplification product (N) over the β-actin internal control (A). In all panels, the upper band is β-actin and the lower band is the amplification product of iNOS.

gene mRNA expression (Crawford *et al*., 2002; Reiner *et al*., 1994; Svetic *et al*., 1991), quantitative RT-PCR assays have not yet been widely used in domestic animal species. To expand the scope and flexibility of the measurement of iNOS mediated responses in chicken, we undertook the development of a rapid, quantitative RT-PCR assay for measuring iNOS gene expression in chicken. Here we described the standard procedure, optimized in our laboratory, to assess chicken iNOS levels with β-actin as an internal control. As for all quantitative techniques, great care must be taken in all optimization steps: the necessary controls to ensure a rough quantitative (semi-quantitative) analysis are described and examples of the analyses of multiple markers and of the data obtained are shown.

It is important to select the appropriate number of cycles so that the amplification product is clearly visible on an agarose gel and can be quantified, but also so that amplification is in the exponential range and has not reached a plateau yet. The optimal number of cycles has to be in the same range for the specific RNA of interest (i.e. iNOS) and the control, in this case β-actin, so that both can be measured on the same gel. According to the results that were obtained in this study (Figure 1), 27 cycles was selected for dual PCR of iNOS and β-actin.

When competition showed as a decrease in intensity of the target RNA in the presence of β-actin (Fig. 3, 1mM MgCl$_2$), different reaction conditions have to be used in terms either of MgCl$_2$ concentration or annealing temperature, or both. At first, quantitation was performed with single primer sets, and only if the amplification products obtained with each iNOS or β-actin primer set had the same intensity as that obtained with both primer sets together, the conditions were considered as optimal (2 and 3 mM MgCl$_2$) (Fig.3). In Fig. 3 it have been illustrated that how iNOS and ?-actin did not compete when combined at 27 cycles in 2 and 3 mM MgCl$_2$. On the other hand, it could be shown as an example that in 1 mM MgCl$_2$, iNOS primers compete with β-actin. The amplification product for iNOS was considerably reduced when co-amplification with both primer sets was performed in 1 mM MgCl$_2$

concentration, and that the competition was completely eliminated when MgCl$_2$ concentration was increased to 2 and 3 mM (Fig. 3).

Co-amplification of the iNOS gene with housekeeping gene provides a quantitative result (Fig. 4). Changes in gene expression level may be monitored, while avoiding sample-to-sample loading variation. This dual quantitative PCR amplification provides a complete solution for RT-PCR iNOS gene expression and identification needs. The RT-PCR described here could readily be modified to quantitate reliably iNOS mRNA expression in any other domestic animal species.

References

1. Ali, S.A., Sart, I., Steinkasserer, A. (1997) Production of PCR mimics for any semiquantitative PCR application. Biotechniques. 22: 1060-62.

2. Aust, G. (2003) Competitive rt-PCR to quantify small amounts of mRNA. Methods Mol.Biol. 249: 31-40.

3. Ausubel, F.M., Kingstone, R., Moore, R., Seidman, D., Smith, J. and Struhl, J. (2002) Current protocols in molecular biology John Wiley and Sons, Inc. New York, USA Unit 4.1.

4. Bredt, D.S., Snyder, S.H. (1994) Nitric oxide: a physiologic messenger molecule. Ann. Rev. Biochem. 63: 175-95.

5. Carding, S.R., Lu, D., Bottomly, K.A. (1992) A polymerase chain reaction assay for the detection and quantification of cytokine gene expression in small number of cells. J.Immunol. Methods 151: 277-87.

6. Coleman, J.W. (2001) Nitric oxide in immunity and inflammation. Int.Immunopharmacol. 1: 1397-1406.

7. Crawford E., Warner, K., Khuder, S., Zahorchak, R. and Willey, J. (2002) Multiplex standardized RT-PCR for expression analysis of many genes in small samples. Biochem. Biophys. Res. Commun. 293:509-16.

8. Djeraba, A., Musset, E., Bernardet, N., Le Vern, Y. and QueAreA, P. (2002) Similar pattern of iNOS expression, NO production and cytokine response in genetic and vaccination-acquired resistance to Marek's disease. Vet. Immunol. Immunopathol.

85:63-75.

9. DuBois, C., Almon, R., Jusko, W. (1993) Molar quantification of specific messenger ribonucleic acid expression in Northern hybridization using cRNA standards. Anal. Biochem. 210: 140-4.

10. Freeman W.M., Walker, S., Vrana, K. (1999) Quantitative RT-PCR: pitfalls and potential. Biotechniques. 26:112-25.

11. Gilliland, G., Perrin, S., Blanchard, K., Bunn, F. (1990) Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA. 87: 2725-29.

12. Griffith, O., Stuehr, D. (1995) Nitric oxide synthases: properties and catalytic mechanism. Annu. Rev. Physiol. 57: 707-36

13. Hampl, V., Herget, J. (2000) Role of Nitric Oxide in the Pathogenesis of Chronic Pulmonary Hypertension. Physiol Rev. 80: 1337-72.

14. Han, B., DuBois, D., Boje, K.S., Almon, R. (1999) Quantification of iNOS mRNA with reverse transcription polymerase chain reaction directly from cell lysates, Nitric Oxide. 3: 281-91

15. Kobzik, l., Bredt, D.S., Lovenstain, C., Drazon, J., Gaston, B., Sugarbaker, D. and Stamler, J. S. (1993) Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. Am. J. Respir. Cell Mol. Biol. 9:371-7.

16. Kroncke, K., Fehsel, K., Suschek, C., Kolb-Bachofen, V. (2001) Inducible nitric oxide synthase-derived nitric oxide in gene regulation, cell death and cell survival, Int.Immunopharmacol. 1: 1407-20.

17. Lamas, S., Marsden, P., Li, G., Tempst, P. and Michel, T. (1992) Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proc. Natl. Acad. Sci. U.S.A. 89: 6348-52.

18. Liew, F. (1995) Nitric oxide in infectious and autoimmune diseases, Ciba Found.Symp. 195: 234-239.

19. Liew, F.Y., Cox, F. (1991) Nonspecific defense mechanism: the role of nitric oxide. Immunol. Today 12: A17-A21.

20. Lin, A.W., Chang, C.C., McCormick, C.C. (1996) Molecular cloning and expression of an avian macrophage nitric-oxide synthase cDNA and the analysis of the genomic5-Flanking Region. J. Biol. Chem. 271: 11911-11919.

21. Melton, D., Krieg, P., Rebagliati, M., Maniatis, T., Zinn, K. and Green, M. (1984) Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. 12: 7035-56.

22. Nathan, C. (1992) Nitric oxide as a secretory product of mammalian cells. FASEB J. 6: 3051-64.

23. Reiner, S., Zheng, S., Corry, D. and Locksley, R. (1994) Constructing polycompetitor cDNAs for quantitative PCR. J. Immunol. Method. 165: 37-46.

24. Svetic, A., Finkelman, F., Jian, Y., Dieffenbach, C., Scott, D., McCarthy, K., Steinberg, A. and Gause, W. (1991) Cytokine gene expression after in vivo primary immunization with goat antibody to mouse IgD antibody. J. Immunol. 147: 2391-7.

25. Van der Veen R. (2001) Nitric oxide and T helper cell immunity. Int.Immunopharmacol. 1: 1491-1500.

آنالیز نیمه کمی جهت ارزیابی بیان ژن iNOS در ماکروفاژهای طیور

غلامرضا نیکبخت بروجنی ۱* مسعود تشفام ۲ حسین حسن‌پور ۳

۱) گروه میکروبیولوژی دانشکده دامپزشکی دانشگاه تهران، تهران ـ ایران.

۲) گروه فیزیولوژی دانشکده دامپزشکی دانشگاه تهران، تهران ـ ایران.

۳) گروه فیزیولوژی دانشکده دامپزشکی دانشگاه شهر کرد، شهر کرد ـ ایران.

چکیده

iNOS بر اثر عوامل مختلف التهابی بیان می‌گردد و به همین دلیل نوع القایی NOS نامیده می‌شود. تنظیم بیان آن در سطح ژن صورت گرفته و میزان بیان آن ارتباط مستقیمی با تولید بالای NO دارد. بررسی بیان iNOS ابزار قدرتمندی برای درک شاخص‌های ملکولی موثر در پاسخ‌های بافتی و سلولی به عوامل خارجی است. در این تحقیق روش نیمه کمی واکنش RT-PCR برای استخراج RNA طحال طیور و اندازه‌گیری بیان iNOS mRNA در هر نمونه طراحی شد. جزئیات روش برای آنالیز میزان بیان ژن شرح داده شده است. β-actin به عنوان کنترل داخلی برای عادی سازی تغییرات میزان RNA تام در بین نمونه‌ها و کارآیی واکنش استفاده شد. افزوده‌سازی همزمان ژن‌های iNOS و ژن‌های کنترل داخلی (β-actin) نتایج کمی را به دست می‌دهد که امکان اندازه‌گیری تغییرات در میزان بیان ژن را فراهم می‌سازد، بی آن که اختلافات مقدار یک نمونه با نمونه دیگر بر آن تاثیر بگذارد.

واژه‌های کلیدی: iNOS mRNA، RT-PCR، بیان ژن، β-actin.

Code 1326

*) نویسنده مسؤول: تلفن: ۰۲۱-۶۱۱۱۷۰۵۷، نمابر: ۰۲۱-۶۶۴۲۷۵۱۷ Email:nikbakht@ut.ac.ir