Biotechnology Journal International

25(4): 32-38, 2021; Article no.BJI.74543
ISSN: 2456-7051
(Past name: British Biotechnology Journal, Past ISSN: 2231–2927, NLM ID: 101616695)

A Study on the Transcriptional Profile of *NOS2* and *IFN-γ* Genes in River Buffalo with Endometritis

Othman E. Othman[1*], Noha M. Osman[1], Nadia A. Abo El-Maaty[1] and Eman R. Mahfouz[1]

[1]Cell Biology Department, National Research Centre, Dokki, Giza, Egypt.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJI/2021/v25i430146
Editor(s):
(1) Dr. Pankaj Kumar, H.N.B. Garhwal Central University, India.
Reviewers:
(1) Asaad Y. Ayied, University of Basrah, Iraq.
(2) Abbas Rafat, University of Tabriz, Iran.
Complete Peer review History: https://www.sdiarticle4.com/review-history/74543

Original Research Article

Received 01 August 2021
Accepted 07 October 2021
Published 11 October 2021

ABSTRACT

Background and Aim: Uterine lumen contamination with bacteria is ubiquitous in buffalo after parturition. Nearly one-third of these infected animals develop endometritis which leads to reduced fertility. The present study aimed to evaluate the expressions of *IFN-γ* and *NOS2* genes in uterine tissue of buffaloes with endometritis and comparing them with those in healthy animals using RT-qPCR

Materials and Methods: Uterine samples were collected from 50 apparently healthy and 50 clinically infected buffaloes. RNA was extracted from the collected buffalo's uteri and cDNA was synthesized from extracted RNA. Quantitative Real Time PCR technique was performed using this synthesized cDNA.

Results: Apparent up-regulation of both genes mRNA expression was recorded in endometritis-infected animals with 8.3-folds for IFN-γ and 9.99-folds for NOS2 (P<0.001).

Conclusion: The upregulation of *IFN-γ* and *NOS2* expression in the uterine tissue of endometritis-infected buffaloes can be used as a scale for measuring the efficiency of drugs used for endometritis treatment.

**Corresponding author: E-mail: othmanmah@yahoo.com;*

Keywords: Gene expression; IFN-γ; NOS2; buffalo; endometritis; QtPCR.

1. INTRODUCTION

Buffalo is one of the most significant livestock in developing countries including Egypt, where it is the main source of milk and meat. Buffalo production represents about 24.5% of the agricultural gross domestic products. Buffaloes occupy a great position in the Egyptian farmers life compared to cattle for their higher milk fat content and longer productive life. There are 3,9 million heads of buffaloes in Egypt providing the Egyptian market with 44% and 39% of milk and red meat, respectively. Any reduction in supply widens the gap between rising population and increasing demand for this essential food. One of the reasons for decrease in buffalo production is a deficiency in fertility [1]. Low reproductive performance in farm animals can be viewed as one of factors contributing to global economic loss [2].

The uterus of mammals is a sterile environment, although it is always open to infection by many microorganisms, especially during coitus or parturition. Inflammation results from bacterial contamination, which can range from pelvic illness to chronic endometritis and infertility. *E. coli* infection of the endometrium has been linked to a reduction in female fertility [3]. The endometrium, which ascends the genital system in animals after parturition, is the first line of defense against bacterial infection [4].

Increased resistance to fertility-related diseases has led to the elimination of some reproductive problems in this economically important species. Immune genes linked to reproductive diseases can be distinguished by how they are expressed in high and low responders [5]. Many studies investigated the impact of postpartum uterine infection on ovarian function, embryo development, and the likelihood of a healthy pregnancy. Fertility diseases cause the greatest economic loss in bovine production, especially in buffalo [6].

Early detection of subclinical endometritis could help dairy farmers save money on buffalo production. Endometritis can be diagnosed using a variety of procedures, including uterine biopsies and swabs, however these approaches might irritate and distort cells [7]. Because immune genes influence inflammatory responses during infection, differences in the expression patterns of immunity-related genes play a significant role in the early diagnosis of subclinical endometritis [8]. The variation in inflammatory genes expression profiles had a significant impact on the early diagnosis of subclinical endometritis in buffalo.

IFN-γ - which is considered the key factor in behavior of cellular immunity - has a different protective functions to increase the immune responses toward different infections. Its immune-regulation effect was exhibited through the enhancement of antigen processing, leukocyte trafficking, encouragement of anti-viral and anti-microbial functions, cellular proliferation and apoptosis [9]. Type 2 nitric oxide synthase (*NOS2*) enzyme has an important role in murder different microbial pathogens through the generation of nitric oxide from the amino acid L-arginine. The enhanced expression of *NOS2* has been related with immune response against infection with pathogens [10-11].

2. MATERIALS AND METHODS

2.1 Sampling

The uteri samples were collected from 100 Egyptian buffaloes, 50 infected with endometritis and 50 apparently normal ones. The uterine samples were collected in slaughterhouse from animals after sacrificing under normal condition without any special requirement, so it is not needed to any ethical permission. Samples were divided to apparently healthy uteri (50n) and clinically infected ones (50n) depending on physical examination and detection of abnormal secretions and inflammation signs in uterine tissues. Bacteriological processing was performed on all uterine samples to confirm the identification detected by visual inspection and to identify bacterial pathogens from clinically diseased samples.

2.2 RNA Isolation and Reverse Transcription

RNA was extracted from uterine tissue using total RNA purification kit (Jena Bioscience, Germany), according to manufacturer's instructions. An aliquot of RNA was diluted in RNase free water to estimate RNA purity and quantity by Nano Drop where the 260/280 nm ratio ranged from 1.9 to 2.1. The DNase-treated RNA was reverse transcribed into first strand cDNA using RevertAid First Strand cDNA

Synthesis kit (Fermantas) according to the manufacturer's instructions.

2.3 Quantitative Real Time PCR (qRT-PCR)

Gene expressions of two tested genes were detected by real-time PCR, which was performed using Rotor-Gene Q system (Qiagen Company). A 25 µl reaction mixture consisted of 12.5 µl SYBR Green PCR Master-Mix (applied Biosciences, USA), 0.5 µl of each primer (10 PMole) (Table 1), 1 µl cDNA (50 ng) and 10.5 µl RNase free water. Cycling was performed using the following conditions: holding at 95°C for 10 min and then 40 cycles with two steps; 30 Sec at 95°C and 45 Sec at the specific temperature of each gene.

2.4 Statistical Analysis

Data from real-time PCR were analyzed using $2^{-\Delta\Delta Ct}$ method [15]. Data were represented as the fold change in target gene expression normalized to a House-Keeping gene. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control gene. The chi-square test was used to evaluate the significant differences in gene expression of tested genes.

3. RESULTS AND DISCUSSION

Buffalo is a significant livestock animal that has aided human survival for over a thousand years especially in developing countries like Egypt. Improving fertility is critical for lowering production costs, where the demand for buffalo's meat is increasing recently due to over-growing population. During selection indexes, the reproductive traits including ovulation, mating and calving-related traits were depreciated to increase livestock genetic capacity [16]. Buffaloes' fertility and reproductive success are greatly influenced by the postpartum era. In this respect, uterine diseases, especially subclinical endometritis, are linked to decreased productivity and reproduction [17]. Uterine infection and inflammation are normally terminated by the uterine normal resistance mechanisms. However, infection can continue in some animals and cause infertility even after clinical signs have faded [18].

Postpartum endometritis is one of the common diseases in dairy animals, like buffalo, leads to economic losses due to increased inter-calving intervals [19]. Determining the immune status of buffalo in relation to the occurrence of endometritis may assist to improve some strategies for effective reproductive management. Despite high frequency of animals can clear the uterine from bacteria, around 30% of infected buffalo showed subclinical endometritis [20].

The infection can spread across the herd due to the difficulty of detecting subclinical endometritis, where animals are bacteria reservoirs despite their safe appearance [8]. Consequently, early identification of animals with subclinical endometritis is regarded as the safest and most effective approach for managing endometritis in buffalo and lowering its economic effects [21]. Some reports declared the association between expressions of immunity genes with the occurrence of clinical or subclinical endometritis in cow [22]. Increased immunity gene expression in cows is thought to be a sensitive predictor of endometritis [23,24,25].

Innate and adaptive immune mechanisms are thought to be the most important factors in controlling infectious diseases. In the equilibrium between these two processes, cytokines play an orchestral function [26]. During inflammation, cytokines are involved in the control of tissue repair [27]. This work aimed to assess the relation between the presence of pathogenic bacteria in uterine of buffaloes and the expression of *NOS2* and *IFN-γ* genes to gain further insight into the effects of uterine infection on the immune system.

Table 1. Information of used primers

Gene	Primer sequence	Tm (°C)	References
IFN-γ	F: TGGAGG ACTTCA AAAAGCTGATT R: TTTATG GCTTTG CGCTGG AT	60	[12]
NOS2	F: GGACAGTAAAGACGTCTCCAGA R: TATGGTCAAACTTTTGGGGTTC	54	[13]
GAPDH	F: CCT GGA GAA ACC TGC CAA GT R: GCC AAA TTC ATT GTC GTA CCA	60	[14]

Normal healthy and endometritis infected buffaloes were tested for gene expression of *IFN-γ* and *NOS2* genes. Data from QRT-PCR were analyzed using the $2^{-\Delta\Delta Ct}$ method. *GAPDH* served as the housekeeping gene to calculate the ΔCt values. Animals infected with endometritis showed an increase in IFN-γ cytokine gene expression compared to uninfected ones. IFN-γ mRNA levels were significantly up-regulated by 8.34 folds (P<0.001) in infected animals compared to uninfected ones (Fig. 1).

On the other hand, NOS2 gene showed increased expression levels in buffalo infected animals compared to normal ones. NOS2 mRNA levels were significantly up-regulated by 9.99 folds with a P value <0.001 (Fig. 2).

Cytokines has as essential role in the defense of animals against the invading of different microbial pathogens [28]. Interferon gamma is one of cytokines which produced in different types of immune cells and has different biological and physiological functions in mammas including the reproduction [29-30]. After infection with pathogens, the immune cells on the cell surface detect the invaded pathogens and activate the production of some cytokines including *IFN-γ* [31]. Our finding in the present study declared the elevation of *IFN-γ* in endometritis-infected buffalo and this result agrees with reports about the production of some cytokines; *TNFα*, *IL1β* and *TNF after the stimulation of* gestational cells with bacteria [32].

The inflammatory mediator nitric oxide expression has a role in the inflammation toxicity. The increased concentration of NO was detected in uterine secretion of endometritis-infected cattle compared with the healthy animals [33]. The high gene expression of *NOS2* in buffalo infected with endometritis was reported in the present study where its expression was increased by about 10 folds in infected animals supporting its role in the inflammation as immunity response against infection with different pathogens. Also, the highly expression of inflammatory factors including different interleukins and NOS2 was reported in cattle to postpartum bacterial infection [13]. Using mRNA analysis, the increased expression of tumor necrosis factor alpha and inducible nitric oxide synthase was detected in cow suffered the subclinical endometritis after repeated breeding [34,35].

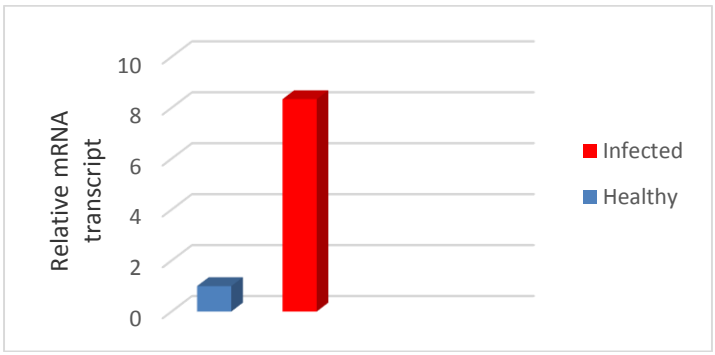

Fig. 1. N-fold change of IFN-γ gene expression in endometritis-infected buffalos compared to healthy animals

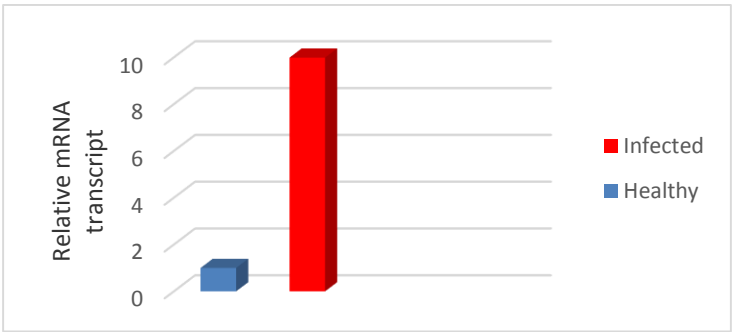

Fig. 2. N-fold change of NOS2 gene expression in endometritis-infected buffalos compared to healthy animals

4. CONCLUSION

In conclusion, upregulation of *IFN-γ* and *NOS2* transcripts in the uterine tissue of buffaloes infected with endometritis indicates uterine inflammation and it can be used as a scale for measuring the efficiency of drugs used for endometritis treatment.

DISCLAIMER

There is absolutely no conflict of interest between the authors of this manuscript and any other scientists or producers, and this research was funded by National Research Centre and personal efforts of the authors without funding from any other agents or funders.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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