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ЕЖЕМЕСЯЧНЫЙ НАУЧНЫЙ ЖУРНАЛ

**Медицинские новости Грузии**

საქართველოს სამედიცინო სიახლენი

**GEORGIAN MEDICAL NEWS**

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**GMN: Georgian Medical News** is peer-reviewed, published monthly journal committed to promoting the science and art of medicine and the betterment of public health, published by the GMN Editorial Board since 1994. GMN carries original scientific articles on medicine, biology and pharmacy, which are of experimental, theoretical and practical character; publishes original research, reviews, commentaries, editorials, essays, medical news, and correspondence in English and Russian.

GMN is indexed in MEDLINE, SCOPUS, PubMed and VINITI Russian Academy of Sciences. The full text content is available through EBSCO databases.

**GMN: Медицинские новости Грузии -** ежемесячный рецензируемый научный журнал, издаётся Редакционной коллегией с 1994 года на русском и английском языках в целях поддержки медицинской науки и улучшения здравоохранения. В журнале публикуются оригинальные научные статьи в области медицины, биологии и фармации, статьи обзорного характера, научные сообщения, новости медицины и здравоохранения. Журнал индексируется в MEDLINE, отражён в базе данных SCOPUS, PubMed и ВИНИТИ РАН. Полнотекстовые статьи журнала доступны через БД EBSCO.

**GMN: Georgian Medical News –** საქართველოს სამედიცინო სიახლენი – არის ყოველთვიური სამეცნიერო სამედიცინო რეცენზირებადი ჟურნალი, გამოიცემა 1994 წლიდან, წარმოადგენს სარედაქციო კოლეგიისა და აშშ-ის მეცნიერების, განათლების, ინდუსტრიის, ხელოვნებისა და ბუნებისმეტყველების საერთაშორისო აკადემიის ერთობლივ გამოცემას. GMN-ში რუსულ და ინგლისურ ენებზე ქვეყნდება ექსპერიმენტული, თეორიული და პრაქტიკული ხასიათის ორიგინალური სამეცნიერო სტატიები მედიცინის, ბიოლოგიისა და ფარმაციის სფეროში, მიმოხილვითი ხასიათის სტატიები.

ჟურნალი ინდექსირებულია MEDLINE-ის საერთაშორისო სისტემაში, ასახულია SCOPUS-ის, PubMed-ის და ВИНИТИ РАН-ის მონაცემთა ბაზებში. სტატიების სრული ტექსტი ხელმისაწვდომია EBSCO-ს მონაცემთა ბაზებიდან.

**WEBSITE**

www.geomednews.com

## К СВЕДЕНИЮ АВТОРОВ!

При направлении статьи в редакцию необходимо соблюдать следующие правила:

1. Статья должна быть представлена в двух экземплярах, на русском или английском языках, напечатанная через **полтора интервала на одной стороне стандартного листа с шириной левого поля в три сантиметра**. Используемый компьютерный шрифт для текста на русском и английском языках - **Times New Roman** (**Кириллица**), для текста на грузинском языке следует использовать **AcadNusx.** Размер шрифта - **12**. К рукописи, напечатанной на компьютере, должен быть приложен CD со статьей.

2. Размер статьи должен быть не менее десяти и не более двадцати страниц машинописи, включая указатель литературы и резюме на английском, русском и грузинском языках.

3. В статье должны быть освещены актуальность данного материала, методы и результаты исследования и их обсуждение.

При представлении в печать научных экспериментальных работ авторы должны указывать вид и количество экспериментальных животных, применявшиеся методы обезболивания и усыпления (в ходе острых опытов).

4. К статье должны быть приложены краткое (на полстраницы) резюме на английском, русском и грузинском языках (включающее следующие разделы: цель исследования, материал и методы, результаты и заключение) и список ключевых слов (key words).

5. Таблицы необходимо представлять в печатной форме. Фотокопии не принимаются. **Все цифровые, итоговые и процентные данные в таблицах должны соответствовать таковым в тексте статьи**. Таблицы и графики должны быть озаглавлены.

6. Фотографии должны быть контрастными, фотокопии с рентгенограмм - в позитивном изображении. Рисунки, чертежи и диаграммы следует озаглавить, пронумеровать и вставить в соответствующее место текста **в tiff формате**.

В подписях к микрофотографиям следует указывать степень увеличения через окуляр или объектив и метод окраски или импрегнации срезов.

7. Фамилии отечественных авторов приводятся в оригинальной транскрипции.

8. При оформлении и направлении статей в журнал МНГ просим авторов соблюдать правила, изложенные в «Единых требованиях к рукописям, представляемым в биомедицинские журналы», принятых Международным комитетом редакторов медицинских журналов - http://www.spinesurgery.ru/files/publish.pdf и http://www.nlm.nih.gov/bsd/uniform_requirements.html
В конце каждой оригинальной статьи приводится библиографический список. В список литературы включаются все материалы, на которые имеются ссылки в тексте. Список составляется в алфавитном порядке и нумеруется. Литературный источник приводится на языке оригинала. В списке литературы сначала приводятся работы, написанные знаками грузинского алфавита, затем кириллицей и латиницей. Ссылки на цитируемые работы в тексте статьи даются в квадратных скобках в виде номера, соответствующего номеру данной работы в списке литературы. Большинство цитированных источников должны быть за последние 5-7 лет.

9. Для получения права на публикацию статья должна иметь от руководителя работы или учреждения визу и сопроводительное отношение, написанные или напечатанные на бланке и заверенные подписью и печатью.

10. В конце статьи должны быть подписи всех авторов, полностью приведены их фамилии, имена и отчества, указаны служебный и домашний номера телефонов и адреса или иные координаты. Количество авторов (соавторов) не должно превышать пяти человек.

11. Редакция оставляет за собой право сокращать и исправлять статьи. Корректура авторам не высылается, вся работа и сверка проводится по авторскому оригиналу.

12. Недопустимо направление в редакцию работ, представленных к печати в иных издательствах или опубликованных в других изданиях.

**При нарушении указанных правил статьи не рассматриваются**.

## REQUIREMENTS

Please note, materials submitted to the Editorial Office Staff are supposed to meet the following requirements:

1. Articles must be provided with a double copy, in English or Russian languages and typed or compu-ter-printed on a single side of standard typing paper, with the left margin of **3** centimeters width, and **1.5** spacing between the lines, typeface - **Times New Roman** (**Cyrillic**), print size - **12** (referring to Georgian and Russian materials). With computer-printed texts please enclose a CD carrying the same file titled with Latin symbols.

2. Size of the article, including index and resume in English, Russian and Georgian languages must be at least 10 pages and not exceed the limit of 20 pages of typed or computer-printed text.

3. Submitted material must include a coverage of a topical subject, research methods, results, and review.

Authors of the scientific-research works must indicate the number of experimental biological species drawn in, list the employed methods of anesthetization and soporific means used during acute tests.

4. Articles must have a short (half page) abstract in English, Russian and Georgian (including the following sections: aim of study, material and methods, results and conclusions) and a list of key words.

5. Tables must be presented in an original typed or computer-printed form, instead of a photocopied version. **Numbers, totals, percentile data on the tables must coincide with those in the texts of the articles.** Tables and graphs must be headed.

6. Photographs are required to be contrasted and must be submitted with doubles. Please number each photograph with a pencil on its back, indicate author's name, title of the article (short version), and mark out its top and bottom parts. Drawings must be accurate, drafts and diagrams drawn in Indian ink (or black ink). Photocopies of the X-ray photographs must be presented in a positive image in **tiff format**.

Accurately numbered subtitles for each illustration must be listed on a separate sheet of paper. In the subtitles for the microphotographs please indicate the ocular and objective lens magnification power, method of coloring or impregnation of the microscopic sections (preparations).

7. Please indicate last names, first and middle initials of the native authors, present names and initials of the foreign authors in the transcription of the original language, enclose in parenthesis corresponding number under which the author is listed in the reference materials.

8. Please follow guidance offered to authors by The International Committee of Medical Journal Editors guidance in its Uniform Requirements for Manuscripts Submitted to Biomedical Journals publication available online at: http://www.nlm.nih.gov/bsd/uniform_requirements.html
http://www.icmje.org/urm_full.pdf
In GMN style for each work cited in the text, a bibliographic reference is given, and this is located at the end of the article under the title "References". All references cited in the text must be listed. The list of references should be arranged alphabetically and then numbered. References are numbered in the text [numbers in square brackets] and in the reference list and numbers are repeated throughout the text as needed. The bibliographic description is given in the language of publication (citations in Georgian script are followed by Cyrillic and Latin).

9. To obtain the rights of publication articles must be accompanied by a visa from the project instructor or the establishment, where the work has been performed, and a reference letter, both written or typed on a special signed form, certified by a stamp or a seal.

10. Articles must be signed by all of the authors at the end, and they must be provided with a list of full names, office and home phone numbers and addresses or other non-office locations where the authors could be reached. The number of the authors (co-authors) must not exceed the limit of 5 people.

11. Editorial Staff reserves the rights to cut down in size and correct the articles. Proof-sheets are not sent out to the authors. The entire editorial and collation work is performed according to the author's original text.

12. Sending in the works that have already been assigned to the press by other Editorial Staffs or have been printed by other publishers is not permissible.

**Articles that Fail to Meet the Aforementioned Requirements are not Assigned to be Reviewed.**

## ავტორთა საყურადღებოდ!

რედაქციაში სტატიის წარმოდგენისას საჭიროა დავიცვათ შემდეგი წესები:

1. სტატია უნდა წარმოადგინოთ 2 ცალად, რუსულ ან ინგლისურ ენებზე,დაბეჭდილი სტანდარტული ფურცლის 1 გვერდზე, 3 სმ სიგანის მარცხენა ველისა და სტრიქონებს შორის 1,5 ინტერვალის დაცვით. გამოყენებული კომპიუტერული შრიფტი რუსულ და ინგლისურენოვან ტექსტებში - **Times New Roman (Кириллица)**, ხოლო ქართულენოვან ტექსტში საჭიროა გამოვიყენოთ **AcadNusx.** შრიფტის ზომა – 12. სტატიას თან უნდა ახლდეს CD სტატიით.

2. სტატიის მოცულობა არ უნდა შეადგენდეს 10 გვერდზე ნაკლებს და 20 გვერდზე მეტს ლიტერატურის სიის და რეზიუმეების (ინგლისურ, რუსულ და ქართულ ენებზე) ჩათვლით.

3. სტატიაში საჭიროა გაშუქდეს: საკითხის აქტუალობა; კვლევის მიზანი; საკვლევი მასალა და გამოყენებული მეთოდები; მიღებული შედეგები და მათი განსჯა. ექსპერიმენტული ხასიათის სტატიების წარმოდგენისას ავტორებმა უნდა მიუთითონ საექსპერიმენტო ცხოველების სახეობა და რაოდენობა; გაუტკივარებისა და დაძინების მეთოდები (მწვავე ცდების პირობებში).

4. სტატიას თან უნდა ახლდეს რეზიუმე ინგლისურ, რუსულ და ქართულ ენებზე არანაკლებ ნახევარი გვერდის მოცულობისა (სათაურის, ავტორების, დაწესებულების მითითებით და უნდა შეიცავდეს შემდეგ განყოფილებებს: მიზანი, მასალა და მეთოდები, შედეგები და დასკვნები; ტექსტუალური ნაწილი არ უნდა იყოს 15 სტრიქონზე ნაკლები) და საკვანძო სიტყვების ჩამონათვალი (key words).

5. ცხრილები საჭიროა წარმოადგინოთ ნაბეჭდი სახით. ყველა ციფრული, შემაჯამებელი და პროცენტული მონაცემები უნდა შეესაბამებოდეს ტექსტში მოყვანილს.

6. ფოტოსურათები უნდა იყოს კონტრასტული; სურათები, ნახაზები, დიაგრამები - დასათაურებული, დანომრილი და სათანადო ადგილას ჩასმული. რენტგენოგრამების ფოტოასლები წარმოადგინეთ პოზიტიური გამოსახულებით **tiff** ფორმატში. მიკროფოტოსურათების წარწერებში საჭიროა მიუთითოთ ოკულარის ან ობიექტივის საშუალებით გადიდების ხარისხი, ანათალების შეღებვის ან იმპრეგნაციის მეთოდი და აღნიშნოთ სურათის ზედა და ქვედა ნაწილები.

7. სამამულო ავტორების გვარები სტატიაში აღინიშნება ინიციალების თანდართვით, უცხოურისა – უცხოური ტრანსკრიპციით.

8. სტატიას თან უნდა ახლდეს ავტორის მიერ გამოყენებული სამამულო და უცხოური შრომების ბიბლიოგრაფიული სია (ბოლო 5-8 წლის სიღრმით). ანბანური წყობით წარმოდგენილ ბიბლიოგრაფიულ სიაში მიუთითეთ ჯერ სამამულო, შემდეგ უცხოელი ავტორები (გვარი, ინიციალები, სტატიის სათაური, ჟურნალის დასახელება, გამოცემის ადგილი, წელი, ჟურნალის №, პირველი და ბოლო გვერდები). მონოგრაფიის შემთხვევაში მიუთითეთ გამოცემის წელი, ადგილი და გვერდების საერთო რაოდენობა. ტექსტში კვადრატულ ფჩხილებში უნდა მიუთითოთ ავტორის შესაბამისი N ლიტერატურის სიის მიხედვით. მიზანშეწონილია, რომ ციტირებული წყაროების უმეტესი ნაწილი იყოს 5-6 წლის სიღრმის.

9. სტატიას თან უნდა ახლდეს: ა) დაწესებულების ან სამეცნიერო ხელმძღვანელის წარდგინება, დამოწმებული ხელმოწერითა და ბეჭდით; ბ) დარგის სპეციალისტის დამოწმებული რეცენზია, რომელშიც მითითებული იქნება საკითხის აქტუალობა, მასალის საკმაობა, მეთოდის სანდოობა, შედეგების სამეცნიერო-პრაქტიკული მნიშვნელობა.

10. სტატიის ბოლოს საჭიროა ყველა ავტორის ხელმოწერა, რომელთა რაოდენობა არ უნდა აღემატებოდეს 5-ს.

11. რედაქცია იტოვებს უფლებას შეასწოროს სტატია. ტექსტზე მუშაობა და შეჯერება ხდება საავტორო ორიგინალის მიხედვით.

12. დაუშვებელია რედაქციაში ისეთი სტატიის წარდგენა, რომელიც დასაბეჭდად წარდგენილი იყო სხვა რედაქციაში ან გამოქვეყნებული იყო სხვა გამოცემებში.

აღნიშნული წესების დარღვევის შემთხვევაში სტატიები არ განიხილება.

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# PRELIMINARY FINDINGS OF TLR2 AND TLR4 EXPRESSION IN PRETERM NEONATES WITH NECROTIZING ENTEROCOLITIS

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## Abstract.

**Objective:** Necrotizing enterocolitis (NEC) is a leading cause of morbidity and mortality in preterm infants. Despite significant advances made in the prevention and treatment of disease so far, there has not been much change in the rate of mortality and morbidity associated with NEC. Although the factors affecting the development of necrotizing enterocolitis are not yet known precisely, prematurity is thought to be the most important risk factor for the development of NEC. This study aims to determine toll-like receptor (TLR) 2 and TLR4 expression levels in preterm neonates.

**Methods:** A total of 50 preterm infants (patient: 42, control: 8) were included in the study. TLR2 and TLR4 expression levels were analysed by the RT-qPCR method.

**Results:** While there was no difference in infants' birth weight (g), gestational age (months), mother's age (years), father's age (years), and WBC ($\square 10^9$/L); HGB (g/dL) and RBC ($\square 10^{12}$/L) were found to be significantly higher in the group with NEC ($p<0.05$). When TLR2 and TLR4 relative gene expression levels of neonates were evaluated (log2), it was determined that there was a significant difference between the two groups (below 1500 g) ($p<0.001$). TLR4 relative expression (2^-ddCt, above 1500 g) was higher in the NEC group than in the healthy group, while TLR2 relative expression (2^-ddCt, above 1500 g) was higher in the healthy group.

**Conclusions:** TLR2 and TLR4 have been shown to have prominent roles in the development of NEC in experimental animal models and it would be significant to support this with human studies/animal models for a better understanding of the disease. Thus, it is recommended that future studies be carried out on experimental models that better replicate the human body, and dietary factors should be examined in detail.

**Key words.** Necrotizing enterocolitis, toll-like receptors, immune system, preterm, neonatal.

## Introduction.

Necrotizing enterocolitis (NEC), a serious gastrointestinal system (GIS) disease, is characterized by intestinal inflammation and necrosis [1-3]. NEC is frequently encountered in preterm and has a high mortality and morbidity rate. Also, it is the leading cause of death because of gastrointestinal disease in preterm newborns and affects 5-12% of very-low-birth-weight newborns [4]. Despite the fact that there have been significant developments in neonatal care and treatment, there has not been a considerable change in the rate of NEC-related mortality and morbidity [5]. The incidence of NEC has been reported to be in the range of 1-5%, the mortality rate as 10-30% and the morbidity rate as 20-40% in neonatal intensive care units [6,7].

Many factors are thought to play a role in the development of necrotizing enterocolitis; however, its exact etiology is not yet fully known [8]. Nonetheless, prematurity is the most important risk factor for the development of NEC, and it is frequently encountered in infants with a birth weight of fewer than 1000 grams [9]. In addition, intestinal ischemia, immature intestinal structure, bacterial proliferation, and formula and enteral nutrition are reported to be associated factors in the development of NEC [10]. The immature intestine leads to a weakened intestinal epithelial barrier, an underdeveloped immune defence, and altered vascular development and tone. The epithelial barrier and underdeveloped immune system can lead to intestinal inflammation and sepsis when exposed to the luminal microbiota shaped by formula feedings, antibiotic exposure, and caesarean section [11]. Therefore, intestinal maturation and colonization (dysbiosis) in preterm infants are considered to be critical factors in the development of NEC [12]. Altered intestinal colonization (especially gram-negative bacteria), which initiates an unstable proinflammatory response in preterm infants, leads to the breakdown of the intestinal mucosal barrier resulting in intestinal necrosis, which is thought to be one of the pathways leading to NEC [13]. The development of NEC is almost exclusively limited to babies born prematurely [14]. NEC usually occurs 8-10 days after birth when gram-negative bacteria and other microorganisms colonize the gut of the premature baby [15]. In addition, infants with NEC have reported lower microbiota diversity and abnormal bacterial colonization [16].

Toll-like receptors (TLRs) are important components of the innate immune system, as they recognize pathogen-associated molecular patterns originating from a variety of microbes. These receptors are examined in two subgroups as transmembrane (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) and intracellular (TLR3, TLR7, TLR8, and TLR9) [16-18]. TLRs have the unique capacity to detect initial infection and are the most potent inducers of inflammatory responses [19]. These molecules have significant roles in the development of various diseases [16]. While TLR4 has critical roles in host defence against inflammation caused by lipopolysaccharides found in most gram-negative bacteria, lipopeptides and other components of gram-positive bacteria have been shown to signal predominantly via TLR2 (along with TLR1 or TLR6) [20].

Studies have shown that TLRs, especially TLR2 and TLR4, have main roles in the pathogenesis of NEC [16,21]. TLR4 is

one of the most important pathways playing a key role in the pathogenesis of NEC and mediates the balance between damage and repair in the premature gut. The TLR4-mediated imbalance between proinflammatory and anti-inflammatory signaling in the premature intestinal epithelium is thought to lead to the development of NEC [22]. TLR4 expression levels decrease shortly after birth in term infants. TLR4 level in term infants does not cause a pro-inflammatory response but allows normal adaptation of small intestinal bacteria in the environment of microbial colonization [23]. In premature infants, however, TLR4 levels remain elevated both before and after birth, due to the normal ontogeny of gene expression. This causes bacterial translocation, systemic sepsis, and NEC development in infants with NEC [23, 24].

Although it is emphasized in the literature that TLR2 and TLR4 are effective in the pathogenesis of infants with NEC, studies are very limited and mostly focused on TLR4. Therefore, this study aimed to determine and compare TLR2 and TLR4 expressions in preterm neonates (with NEC) with healthy preterm neonates.

## Materials and methods.

### Subjects:

A total of 50 preterm neonates (before 37 weeks of pregnancy and <2500 g), including 42 neonates with NEC and 8 healthy neonates, were included in the study. All the examined neonates were divided into 2 groups: the patient and the control group. They, in turn, were divided into 2 subgroups, children with a body weight of more than 1500 g and a weight of less than 1500 g.

The patients were selected from children undergoing treatment in the Department of Anesthesiology, Resuscitation and Intensive Care of New-borns and department of Premature babies of the Scientific Research Institute of Paediatrics named after K. Farajova between February 2022 and July 2022. The control group were selected from Maternity Hospital No. 7 based on the consultation by responsible medical doctors that they were healthy. Considering the premature birth of children under our control, the volume of blood taken was 0.5 ml. Genetic tests were carried out at the Institute of Genetic Resources in Baku and in a private laboratory.

The study protocol was approved by the Ethics Committee of a university on 04.02.2022 (report number: 22). In addition, consent was received from the families.

**The determination of TLR2 and TLR4 expression levels:**

Expression levels of genes were determined by the Real time-polymerase chain reaction (RT-qPCR) method by following the steps below. All stages were carried out by taking into account the principles of temperature and contamination.

**RNA isolation:** A total RNA isolation kit was used to ensure high quality total RNA extraction (miRNeasy Serum/Plasma Kit, Qiagen, Germany). The procedures were carried out following the manufacturer's protocol.

The nucleic acid loads (ng values) of the samples obtained from the total RNA extraction process are fixed at 1000 ng to be used in the next steps of the study. The total RNA amounts of the samples were determined using a microvolume spectrophotometer device (Colibri Titertek Berthold, Germany). After the device was started, the nucleic acid tab was selected, and the parameters were adjusted so that the sample type RNA-40 and the light path length were automatic. After the device cover was opened, 2 µl of the elution buffer (Vivantis Total RNA Extraction Kit, Malaysia) was added and blanking was performed. The RNA measurement process was completed by taking 2 µl of the samples, respectively, and using the measurement option.

**Reverse transcriptase process (cDNA):** The reverse transcriptase process was performed with Oligo (dT) and Random Hexamer primers for the detection of pre-mRNA and mature-mRNA structures. By means of these primers, both mature and immature mRNA chains are synthesized by the Reverse Transcriptase enzyme from the opposite copy of the mRNA sequence.

OneScript Plus Reverse Transcriptase (ABMgood, Canada) was used for cDNA processing with total RNA from all samples. Using 11.5 µl of each of the total RNAs, cDNA processing was performed in accordance with the following protocol.

For the first step, the components given in Table 1 were prepared in a sterile tube on ice.

***Table 1.*** *cDNA reaction mixture.*

| Components | Quantity | Final concentration |
|---|---|---|
| Total RNA | 2 µl | 50-500 µg/reaction |
| dNTP (10M) | 1 µl | 500 µM |
| Oligo (dT) (10 µM) | 1 µl | 0,5 µM |
| Random Hexamer (10 µM) | 1 µl | 0,5 µM |
| Nuclease-free $H_2O$ | Complete up to 14,5 µl | - |

The primer was denatured for 5 min at 65°C. Tubes removed from the device were incubated on ice for 1 min and a short spin was performed. The remaining components were added as given in Table 2.

***Table 2.*** *Reverse transcriptase mixture.*

| Components | Quantity | Final concentration |
|---|---|---|
| 5X RT buffer | 4 µl | 1X |
| OneScript Plus RTase (200 U/µl) | 1 µl | 200 U / reaction |
| RNaseOFF Ribonuclease Inhibitor (40 U/µl) | 0,5 µl | 20 / reaction |

20 µl of mRNA component was incubated at 50 °C for 15 minutes. At the end of the reaction, the enzyme was inactivated at 85 °C for 5 minutes, and the cDNA products were stored at -20 °C.

**SybrGreen-based RNA gene expression:** The cDNA chains created with mRNA sequences were used in relative expression analysis. For each of the samples belonging to the patient and control groups, the relevant mRNA and the sequences of the appropriate reference gene are reproduced with an RT-PCR device. As a result of the process, the data received from the device is evaluated by the $2^{-(\Delta\Delta Ct)}$ method. In studies performed over 40 cycles, the threshold value (Ct, Cp, Cq) taken from the point where the fluorescence value exceeded the threshold value (intersects the threshold line) was used in the calculations.

Genes (TLR2 and TLR4) to be used in the study were supplied ready-made (Biomers, Germany) and beta-actin (ACTB)

(Biomers, Germany) was used as a housekeeping gene. In order to determine the gene expression levels, the binding temperatures were determined by optimizing the primer sets of which ABI 7500 FAST (Thermo USA) designs were completed using BrightGreen qPCR MasterMix (ABM, Canada) (Table 3). Appropriate Tm setups were performed for each gen region and cycle curves and melting curve data were examined. The data obtained after completing the cycle were evaluated using the 2-(ΔΔCt) method relative to the housekeeping gene ACTB [25].

**Statistical analyses:** The data obtained from the study were analysed using the SPSS 23.0 program (Statistical Package for Social Sciences, Inc., Chicago, IL, USA). Results are shown as mean ($\bar{X}$)±standard deviation (SD). Statistical significance level was accepted at the 95% confidence interval, $p<0.05$.

Based on the normality tests for gene expression levels, within-group distributions were examined, and it was determined that the distribution was not normal in all gene groups. However, the characteristics of neonates were normal according to the normality test. Therefore, the Mann Whitney U Test and Independent Sample T-Test were used for pairwise comparisons of the groups.

## Results.

A total of 50 neonates (21 boys, 29 girls) 42 with NEC and 8 healthy, were included in this study. Some characteristic information of neonates was given in Table 4. The birth weight of the neonates in the control group (1782.5±610.22 g) was higher than the patient group, but this difference was not statistically significant ($p>0.05$). The gestational ages of both groups were close to each other ($p>0.05$), and there was no difference between the groups in terms of mother's and fathers' ages ($p>0.05$). HGB, RBC, and WBC levels of neonates with NEC were 12.7±2.96 g/dL, 3.9±0.75 □$10^{12}$/L and 12.7±8.67 □$10^{9}$/L, respectively; in the control group, it is 10.1±1.59 g/dL, 3.1±0.47□$10^{12}$/L and 13.6±8.45 □$10^{9}$/L, respectively. While there was no significant difference between the two groups in terms of WBC values, the RBC HGB values of neonates with NEC were found to be higher than those without NEC ($p<0.05$).

Almost half of the neonates (42%) were born from the first pregnancy, and 58% of them were born from repeated pregnancies. Seven (14%) pregnancies occurred as a result of extracorporeal insemination after long-term infertility. While 24 (48%) neonates were born by vaginal delivery, the other neonates were born by caesarean delivery. Breast milk began to be given to 19 neonates in the I stage on average on the 5th day, 7 neonates in the II stage on the 8th-9th day, and 4 children in the III Stage (57 %) after the 9th day. It was found that 29 neonates (58%) with NEC were fed with formula from the first days of their life. The number of babies receiving only breast milk from the first day of their life was only 2 (5%) (These data are not shown in the table).

Real-time PCR analyses were performed for TLR4 and TLR2 gene regions in subgroups above 1500 g and below 1500 g of NEC and control groups (Table 5). While TLR4 gene expression level was -0.24±2.65 in neonates above 1500 g in the NEC group, it was 0±3.59 in the control group ($p>0.05$). It was observed that TLR2 gene expression level was higher in the control group, but this difference was not statistically significant ($p>0.05$). In the group below 1500 g, both TLR4 and TLR2 gene expression levels were found to differ between the groups ($p<0.05$). TLR4 gene expression level was -4.81±4.01 in the patient group and 0±1.07 in the control group. TLR2 gene expression level was -5.11±3.27 in the patient group and 0±0.78 in the control group ($p<0.05$).

## Discussion.

According to our best knowledge, this is the first study that evaluates the relationship between NEC and TLR2 and TLR4 in preterm neonates. TLRs are members of the type-1 transmembrane receptor family and were first described

***Table 3.*** *Primers were used in the study and their annealing temperatures.*

| Primer Name | Sequence (5'->3') | Length | Tm °C | Amplicon length |
|---|---|---|---|---|
| TLR2-hsa-Algn-F | ACTGGACAATGCCACATACTT | 21 bp | 57 °C | 99 bp |
| TLR2-hsa-Algn-R | CACAAGACAGAGAAGCCTGAT | 21 bp | | |
| TLR4-hsa-Algn-F | AATAGCAACCAACAGTGT | 18 bp | 57 °C | 190 bp |
| TLR4-hsa-Algn-R | CAAGGAGGAAGCATGATT | 18 bp | | |
| ACTB_ej_hsa_F | TGAAGATCAAGATCATTG | 18 bp | 57 °C | 179 bp |
| ACTB_ej_hsa_R | TAACGCAACTAAGTCATA | 18 bp | | |

***Table 4.*** *Some characteristics of neonates.*

| Variables | Patient (n:42) | Control (n:8) | p |
|---|---|---|---|
| | Mean±SD | Mean±SD | |
| Birth weight (g) | 1673.1±546.96 | 1782.5±610.22 | 0.613 |
| Gestational age (week) | 32.4±2.77 | 32.7±3.33 | 0.772 |
| Mother's age (year) | 29.6±7.43 | 30.0±7.07 | 0.888 |
| Father's age (year) | 33.7±6.69 | 30.0±3.16 | 0.285 |
| HGB (g/dL) | 12.7±2.96 | 10.1±1.59 | **0.040** |
| RBC (□$10^{12}$/L) | 3.9±0.75 | 3.1±0.47 | **0.005** |
| WBC (□$10^{9}$/L) | 12.7±8.67 | 13.6±8.45 | 0.771 |

*$p<0.05$; Independent Sample T-Test*

***Table 5.*** *TLR2 and TLR4 relative gene expression levels of neonates (log2).*

| | **Patient (n:42)** | **Control (n:8)** | p |
|---|---|---|---|
| TLR4 | -2.20±3.98 | 0±2.78 | **<0.001** |
| TLR2 | -2.84±3.45 | 0±2.75 | **<0.001** |
| | **Patient (n:24)** | **Control (n:5)** | p |
| | Body weight above 1500 g | | |
| TLR4 | -0.24±2.65 | 0±3.59 | 0.891 |
| TLR2 | -1.22±2.58 | 0±3.59 | 0.375 |
| | **Patient (n:18)** | **Control (n:3)** | |
| | Body weight below 1500 g | | |
| TLR4 | -4.81±4.01 | 0±1.07 | **<0.001** |
| TLR2 | -5.11±3.27 | 0±0.78 | **<0.001** |

in humans in the 1990s [26]. TLR2 and TLR4, which are important members of this family, have been shown to have important roles not only in NEC but also in the pathogenesis of other diseases [27]. However, the mechanisms behind the pathogenesis of NEC and its association with TLR2 and have4 has not been fully understood yet.

TLR4 can recognize bacterial lipopolysaccharide, and its activation mostly results in the synthesis of pro-inflammatory cytokines and chemokines such as TNF-α, interleukin-1, and interferon-γ [28,29]. On the other hand, TLR-2 can identify gram-positive bacteria's peptidoglycan and lipoteichoic acid. Also, its co-expresses with CD14, improving the identification of lipopolysaccharide in gram-negative bacteria's cell walls [30]. Synthetic TLR agonists have recently emerged as a potential therapeutic aim for preferentially stimulating T-helper 1 (Th1) or Th2 immune responses [26]. With these developments, the functions of these receptors related to human health are gaining importance day by day. Therefore, in this study, TLR2 and TLR4 gene expression levels of preterm neonates with or without NEC were studied.

The expression of TLR2 and TLR4 in intestinal epithelial cells after mucosal injury was studied in the rat NEC model [21]. TLR2 and TLR4 mRNA expressions increased in rats with NEC, the combination of immaturity, protein-rich formula, and a hypoxia-reoxygenation procedure was found to cause pathological mucosal damage consistent with NEC, as well as TLR-2 overexpression correlated with the severity of mucosal damage [21]. Similarly, Zhou et al. [31] investigated the mRNA expression of TLR-2 and TLR-4 in the neonatal rat model of NEC. The NEC neonatal rats demonstrated mucosal injury and upregulated mRNA and protein expression of TLR-2, TLR-4, and caspase-3 in the ileum and colon when compared to the normal control. In a different rat model of NEC, the researchers have shown that the expression of TLRs and cytokines (both pro-inflammatory and anti-inflammatory) in the intestine precedes histological injury in the group with NEC [32]. So, it has been clarified that TLR-2 and TLR-4 have a significant role in NEC as well as intestinal immune activation precedes tissue injury [32]. In our study, as a result of the analysis of relative expression (2^-ddCt) of TLR2 and TLR4 (above 1500 g), we found that neonates with NEC had higher TLR4 expression than healthy neonates but TLR2 expression was higher in healthy neonates. It has been stated that prematurity may have an initiating role in NEC with the immaturity of the mucosal barrier [21]. The fact that the number of neonatal in the control group in our study was not sufficient however, since both groups consisted of premature neonates, the results of the analysis might have been similar.

TLR2 and TLR4 are overexpressed in human fetal enterocytes, while expression of TLR2 and TLR4 is very low or absent in healthy adult intestinal epithelium [33]. It has been reported that the expression of TLR2 and TLR4 is altered in infants with NEC [34]. On the other hand, in a study, nine single nucleotide polymorphisms in eight important genes in TLR signalling including TLR2 and TLR4 were analysed in premature infants. Authors reported that there was no significant association between single nucleotide polymorphisms (in TLR2 and TLR4) and NEC [35].

While a well-functioning immune system is essential for surviving, optimal nutrition has very specific roles in developing and maintaining an effective immune system throughout life [36]. All cells, including those in the immune system, require adequate and proper nutrition to function optimally [36]. It has been reported that some foods (formula) or nutrients (saturated and long-chain omega 3 polyunsaturated fatty acids) can interfere with TLR2 and TLR4 activation and thus improve/badly affect the inflammatory signal induced by TLR2 and TLR [31,32,37]. While saturated fatty acids can induce inflammation by activating the TLR4 signaling pathway, on the contrary, long-chain omega 3 polyunsaturated fatty acids (eicosapentaenoic acid and docosahexaenoic acid) can show anti-inflammatory effects by inactivating the TLR4 signaling pathway [37].

A couple of studies examined the effect of different components such as probiotics, glutamine, breastmilk, and formula on the TLRs expression [32] Liu et al. [32] reported that cow milk formula feeding is a proinflammatory stimulation, but formula feeding had a small impact on TLRs expression. On the other hand, in a study comparing different feeding methods in rats with NEC, the most severe gross lesions (necrosis) were observed in rats fed protein-rich formula, while it was reported that although there were haemorrhages in the intestinal wall, there was no necrosis in the rat-milk fed group [21]. The same study showed that there was a gradual decrease in TLR4 expression level in rat-milk-fed rats compared to formula [21]. Glutamine may protect the intestinal tract of preterm neonatal rats with NEC by downregulating the expression of TLR-2 and TLR-4 in the intestines [31]. According to reports published, *Bifidobacterium bifidum* has significant roles not only in the prevention of NEC

but also in improving some parameters related to the disease [38,39]. TLR-2 was activated in the intestinal epithelium of neonatal rats with NEC after *B. bifidum* OLB6378 (5□106 CFU) is taken orally [39]. It is emphasized that probiotics can be effective in reducing both mortality and incidence in infants with NEC, especially for ones below 1500 g, but further studies are needed to understand the mechanism of probiotics in NEC [40]. Considering the significant roles of breastfeeding, formula, and other nutrients in TLR signaling, it is recommended that future studies should focus on these topics in detail.

## Conclusion and Study Limitations.

In the present study, we could not follow up on the breastfeeding and formula intake status of neonates in detail, which is one of the most important limitations of our study. Additionally, the number of neonates in the control group was insufficient owing to limited hospital access. Hence, the sample size is small, and some differences may have been missed due to lack of statistical power. For this reason, it is recommended that future studies should be conducted with a larger sample along with the nutritional status. On the other hand, most of the studies in literature were carried out in animal models. This study was successfully conducted in a vulnerable group (preterm neonates with NEC), and we believe that it will contribute to the literature.

Although the information obtained in recent studies on understanding the pathogenesis of NEC is promising, there are still many points that need to be clarified such as diagnosis and treatment. Especially the data obtained in experimental animal models show that many factors may play a role in the pathogenesis of NEC. In addition, it is known that diet-related modulations can positively alter the expression of TLRs. In this context, the most emphasized dietary components are fatty acids (especially omega 3) and probiotics. In addition, NEC may result in changes in the intestinal microbiota by causing dysbiosis or barrier dysfunction. It has been known for many years that the most effective factor to modulate intestinal microbiota is diet. Considering both the inflammatory parameters and their impacts on the intestinal microbiota, it is obvious that future research should focus, especially on the nutritional dimension. Finally, it is recommended to create NEC experimental animal or in vitro models that more closely resemble and accurately replicate the organism in order to better understand NEC.

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