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Down-regulation of neuronal form of Nitric oxide synthase in the Nurse cell of *Trichinella spiralis*

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Summary

The free radical nitric oxide (NO) and Ca^{2+} are critical regulators of skeletal muscle exercise performance and fatigue. The major source of NO in skeletal muscle cells is the neuronal form of the enzyme Nitric oxide synthase (nNOS). One of the most peculiar characteristics of the Nurse cell of *Trichinella spiralis* (*T. spiralis*) is the complete loss of the contractile capabilities of its derivative striated muscle fiber. The aim of the present study was to clarify the expression of nNOS protein and mRNA in striated muscles during the muscle phase of *T. spiralis* infection in mice. Muscle tissue samples were collected from mice at days 0, 14, 24, and 35 post infection (d.p.i.). The expression of nNOS was investigated by immunohistochemistry, and the expression levels of mRNA of mouse Nitric oxide synthase 1 (*Nos1)* by real-time PCR. The presence of nNOS protein was still well observable in the disintegrated sarcoplasm at the early stage of infection. The cytoplasm of the developing and mature Nurse cell showed the absence of this protein. At least at the beginning of the Nurse cell development, *Trichinella* uses the same repairing process of skeletal muscle cell, induced after any trauma and this corroborates very well our results concerning the nNOS expression on day 14 p.i. At a later stage, however, we could suggest that the down-regulation of nNOS in the Nurse cell of *T. spiralis* either serves a protective function or is an outcome of the genetic identity of the Nurse cell.
Keywords: nNOS; Nurse cell; skeletal muscle; *Trichinella spiralis*

Introduction

Trichinellosis is a food-born zoonosis caused by parasitic nematodes that belong to the *Trichinella* genus. The disease starts in the small intestine where *Trichinella* reproduces. Through the blood stream and the lymph, the newborn larvae travel all over the infected organism. Among the different tissues, however, only the cross-striated muscles are susceptible to accommodate them. After penetrating the sarcolemma, the *Trichinella* larvae induce extreme genetic, morphological, functional, and biochemical changes in the invaded muscle cell. As a result, the occupied portion of the muscle fiber transforms into a structure called a Nurse cell, which is capable of supporting the parasite for years (Capó & Despommier, 1996; Despommier, 1998).

The Nurse cell of the parasitic nematode *Trichinella spiralis* (Owen, 1835) is quite an interesting experimental model of myopathy of an unusual nature. This structure depletes completely the contractile properties of its derivative tissue, due to the irreversible loss of the contractile proteins actin and myosin and the consequent exchange of the cytoplasm (Jasmer, 1993; Despommier, 1998; Matsuo *et al*., 2000). However, it remains well integrated and stable within the surrounding healthy, functional, and highly contractile environment.

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Table 1. The full names of the investigated genes and their primers sequences used in this study.

Gene	Abbreviation	Species	Accession number	Primers sequences (5`-3`)	Product size (bp)
Glyceraldehyde 3-phosphate dehydrogenase	*Gapdh*	*Mus musculus*	NM_001289726, transcript variant 1	TCCTCGTCCCGTAGACAAAATG – F AATCTCCACTTTGCCACTGC – R	103
Nitric oxide synthase 1	*Nos1*	*Mus musculus*	NM_008712.3	CCAGCCAAAGCAGAGATGAAAG – F TCCCCCACAGATCATTGAAGAC – R	121
Expansion segment V	ESV	*Trichinella spiralis*	*	GTTCCATGTGAACAGCAGT – F CGAAAACATACGACAACTGC – R	173

*Zarlenga *et al*., 2001.

The free radical nitric oxide (NO) and Ca^{2+} are critical regulators of skeletal muscle exercise performance and fatigue. The major source of NO in skeletal muscle cells is the neuronal form of the enzyme Nitric oxide synthase (nNOS), which is a component of the subsarcolemmal dystrophin-associated glycoprotein complex (DGC). nNOS catalyzes the synthesis of NO upon excitation, which consequently interacts with the ryanodine receptor 1 Ca^{2+} release channel (RyR1) at the sarcoplasmic reticulum (SR). Once released in the sarcoplasm, Ca^{2+}binds to the troponin C subunit (TnC) on actin and the contraction is activated. In the relaxation phase of the myofiber, Ca^{2+} is pumped back into SR channels where it is stored in association with calsequestrin (Berchtold *et al*., 2000; Percival, 2011).

Considering the contractile properties forfeit of the Nurse cell due to the irreversible loss of actin and myosin, the goal of this research was to investigate the fate of nNOS as a main regulator of contraction in mice skeletal muscles infected with *T. spiralis*.

Material and Methods

Parasites, invasion, sample collection and tissue preparation

Infective *Trichinella spiralis* larvae (code ISS03) were isolated from previously invaded laboratory albino mice (*Mus musculus musculus*), between the 30th and 40th day post infection (d.p.i.) according to a routine protocol, as already described (Milcheva *et al*., 2022). Twenty male white laboratory mice, 6 – 8 weeks old, were divided into two groups – five uninfected and fifteen infected, and the mice from the infected group were inoculated with 500 infective *T. spiralis* larvae *per os.* The animals (five per group) were euthanized at day 0, 14, 24, and 35 post infection (d.p.i.) and skeletal muscle specimens (front and hind limbs, pectoral and gluteal muscles) were excised and fixed with freshly prepared modified methacarn fixative according to Cox *et al*. (2006). After processing the specimens were embedded in paraffin.

Immunohistochemistry

Parallel tissue sections from 14, 24, and 35 d.p.i., 5 µm thick, after deparaffinization were rehydrated and the endogenous peroxidase activity was blocked by 0.3 % H_2O_2 treatment for 5 min. Then the slides were incubated overnight at 4°C with polyclonal rabbit anti-nNOS/NOS Type 1 antibody (BD Transduction Laboratories™, Franklin Lakes, NJ, USA) diluted 1:50 with Dako Real antibody diluents (Agilent Technologies). The reaction was visualized with EnVision™anti-rabbit polymer conjugated with horseradish peroxidase (Agilent Technologies, Santa Clara, CA, USA), developed with 3,3`-diaminobenzidine (DAB) (Agilent Technologies) and the sections were counterstained with hematoxylin. The healthy and non-occupied sectors in each section served as a control. In parallel, negative control specimens were incubated with Dako Real antibody diluent (Agilent Technologies) instead of the antibody. All washings of the slides between the individual staining steps were performed with phosphate-buffered saline (PBS). Additional sections were routinely stained with hematoxylin and eosin (H&E) for basic morphological assessment. The entire surface of each tissue section was evaluated by three independent observers using Nikon Eclipse 80í light microscope (Nikon, Tokio, Japan).

Molecular biology studies

All experimental procedures concerning this chapter were already described in full detail (Milcheva *et al*., 2023). In brief, the expression of mRNA of mouse Nitric oxide synthase 1 (*Nos1*) was evaluated by real-time RT-PCR in tissue samples from mouse skeletal muscle collected at 0, 14, 24, and 35 d.p.i. The levels of expressions were estimated via normalization versus the expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) as a reference gene. The infection of the samples was confirmed by end-point PCR of the Expansion Segment V (ESV) of *T. spiralis* (Zarlenga *et al*., 2001), as already described in detail (Milcheva *et al.,* 2023). Briefly, genomic DNA from six paraffin sections from all samples was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden Germany). Genomic DNA from *T. spiralis* infectious larvae was isolated as a positive control, using NZY Tissue gDNA Isolation Kit (NZYTech, Lisboa, Portugal). All isolations were performed according to the provided protocols of the producers. The yield and purity of the collected gDNA were measured using S-300 Spectrophotometer (Boeco, Hamburg, Germany). Hot start PCR was designed on approximately 100 ng gDNA as a template by using Veriti thermoblock (Applied Biosystems of Thermo Fisher Scientific) under standard PCR conditions (Milcheva *et al*., 2019). The products of amplification were visualized on 2.5 % agarose

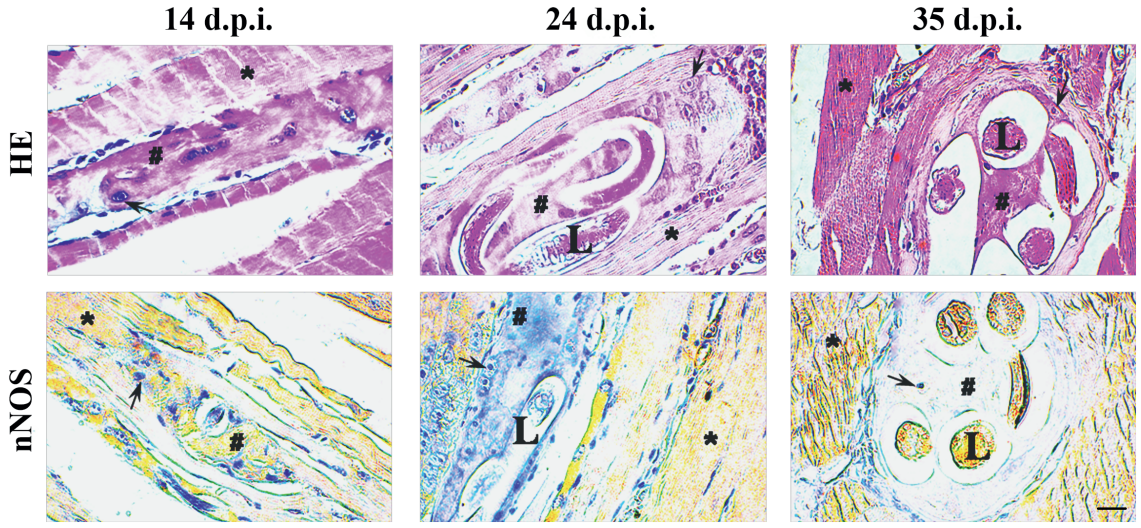

Fig. 1. Immunohistochemistry. Modified methacarn fixed sections from mouse skeletal muscles with *Trichinella spiralis* at days 14, 24, and 35 post invasion (d.p.i.) were stained with polyclonal rabbit anti-nNOS/NOS Type 1 antibody. Parallel sections were subjected to H&E staining to facilitate the histological orientation. The brown colour indicates a positive immunohistochemical reaction, hashtag indicates the occupied sarcoplasma, star – non-occupied skeletal muscle fibre, arrow – enlarged nucleus, L – larva. H&E, anti-mouse peroxidase conjugate, DAB. Obj. magn. – 20x, scale bar 20 μm.

gel supplemented with Simply Safe nucleic acid stain (EurX®, Gdansk, Poland) versus 100-1000 bp DNA Ladder (EurX), and the gels were photographed with a gel documentation system Vision (Scie-Plas Ltd, Cambridge, UK). The primers used for gene expression analyzes were designed using the NCBI Blast Tool (Ye *et al*., 2012) in a way to span at least one intron sequence. The full names of investigated genes, the accession numbers of their reference sequences, the primer sequences, and the size of the amplified products are shown in Table 1. The oligonucleotides were purchased from HVD BiotechVertriebs (Vienna, Austria).

Statistical analysis of the gene expression quantification

Statistical analysis of the data was performed using GraphPad Prism 5.03 software (San Diego, CA, USA). Non-parametric one-way analysis of variance (Kruskal-Wallis test) with Dunn`s Multiple Comparison Test (significance level 0.05) was computed to detect statistically significant differences between the Ct values of the qPCR products between the control and infected samples, and the results were interpreted as follows: $P < 0.001$ = highly significant (***), $P < 0.01$ = very significant (**), $P < 0.05$ = significant (*).

Ethical Approval and/or Informed Consent

All animal experiments were performed in compliance with Regulation № 20/01.11.2012 on the minimum requirements for the protection and welfare of experimental animals and the requirements for the sites for their use, breeding, and/or delivery, issued by the Ministry of Agriculture and Food of Republic of Bulgaria.

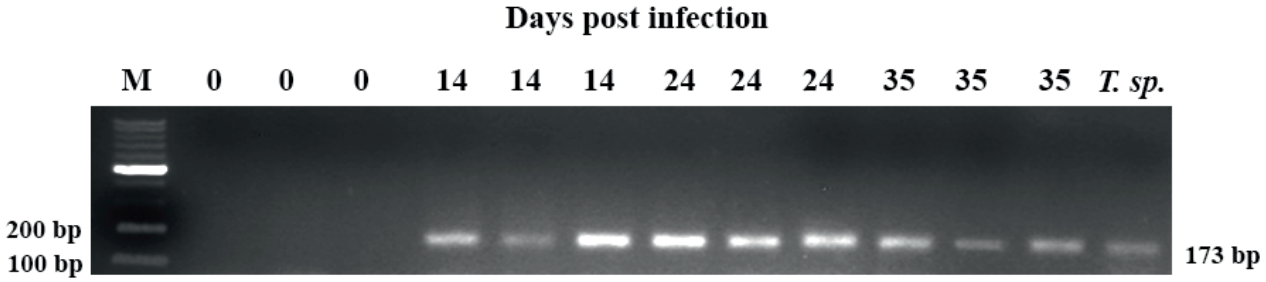

Fig. 2. Agarose gel analysis of *Trichinella spiralis* ESV fragment PCR. Polymerase chain reaction was performed on modified methacarn fixed mouse skeletal muscle tissue sections, selected on days 0, 14, 24, and 35 after *T. spiralis* invasion. Genomic DNA from *T. spiralis* infectious larvae was used as a positive control sample. Presence of 173 bp fragment of expansion segment V of the *T. spiralis* genome was detected only in the mouse samples collected on days 14, 24 and 35 after the invasion. The photograph is representative of three randomly selected samples from each experimental group.

Results

Expression of nNOS protein in the Nurse cell of T. spiralis

The routine histology showed affected muscle cells with typically enlarged and centralized nuclei in disintegrated sarcoplasm at day 14 p.i. and well distinguished Nurse cells at different stages with inside coiled *Trichinella* larvae at days 24 (developing stage) and 35 (mature) p.i. (Fig. 1).

In healthy mouse muscle fibers, nNOS showed regular sarcoplasmic distribution. At day 14 p.i. the expression of the protein was still well presented in the disintegrated sarcoplasm, however, the cytoplasm of the developing and mature Nurse cells showed the absence of nNOS (Fig. 1).

Down-regulation of Nos1 in skeletal muscles infected with T. spiralis detected with real-time RT-PCR

T. spiralis infection was verified in all experimental skeletal muscle

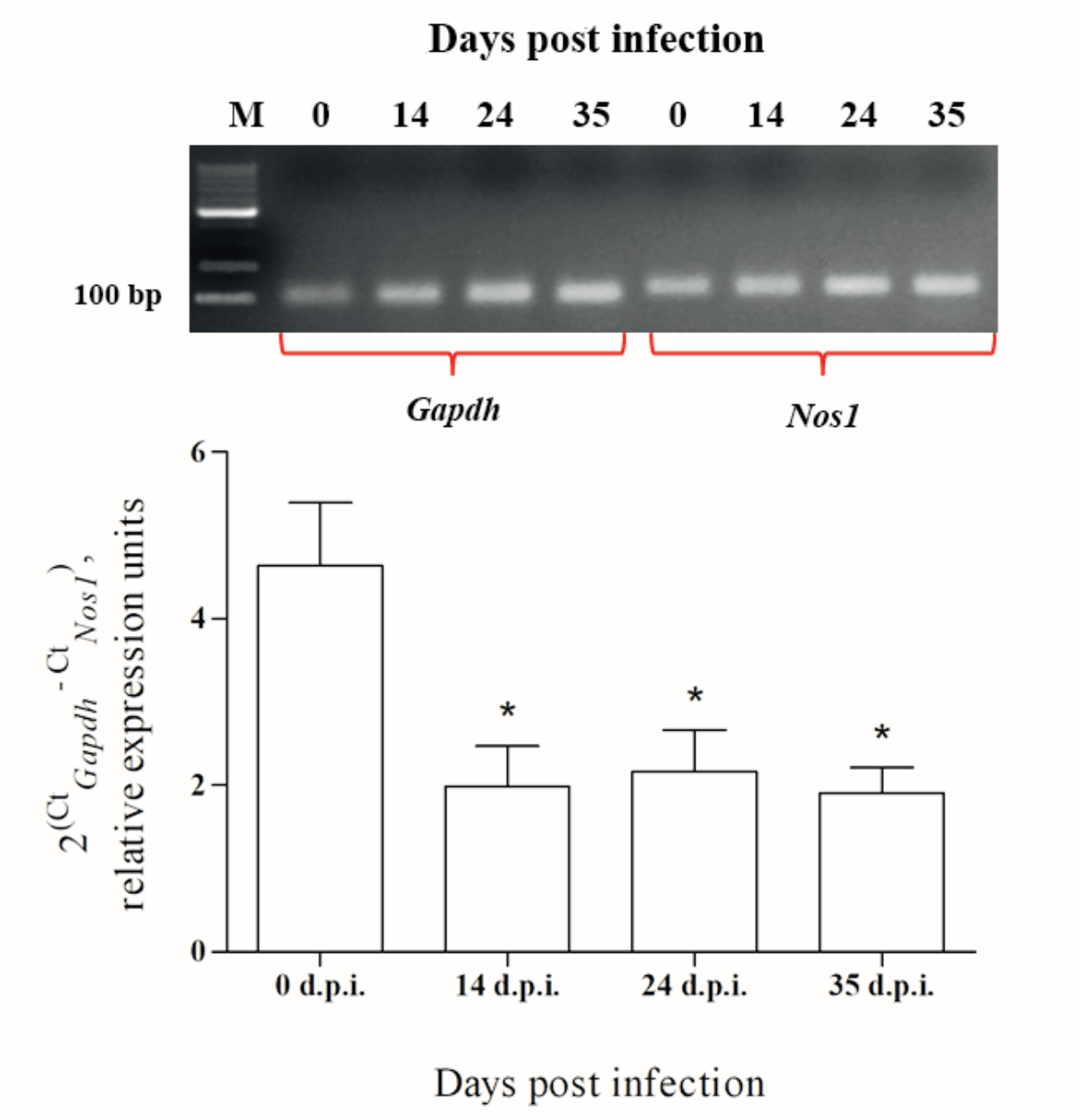

Fig. 3. Expression of mouse Nitric oxide synthase 1 (*Nos1*) analysed by realtime RT-PCR in modified methacarn fixed mouse skeletal muscle tissue sections, selected on days 0, 14, 24, and 35 after *T. spiralis* invasion. The graphs show the relative quantification of the gene expressions calculated by the ΔΔCt method versus glyceraldehyde phosphate dehydrogenase (*Gapdh*) as a reference gene from five individual samples in triplicate. The bars show the standard error of the mean. The products of amplification were loaded on 2.5% agarose gel versus Perfect 100-1000 bp DNA Ladder.

samples by the amplification of the specific Expansion Segment V fragment. The genomic DNA of *T. spiralis* served as a positive control. All non-infected skeletal muscle samples were negative (Fig. 2).

The relative expression analysis of the qPCR data detected slight down-regulation ($P < 0.05$) of *Nos1* in skeletal muscles at days 14, 24, and 35 p.i. in comparison with the non-infected muscle samples (Fig. 3).

Discussion

Most infections of muscle ultimately result in the death of the infected muscle fiber and often have crucial consequences on the organism overall (Crum-Cianflone, 2008). Therefore, the relationship of the *T. spiralis* larva with its host cell is a unique evolutionary event enabling the Nurse cell to remain alive for years, supporting the life of the parasite.

Some of the most prominent morphological changes within the portion of the skeletal muscle fiber occupied by a newborn larva of *T. spiralis*, in the time course of its de-differentiation to a Nurse cell, include complete loss of contractile elements, enlargement of nuclei, hypertrophy of SR, and development of host-derived collagen capsule isolating the Nurse cell from the surrounding non-affected area (Despommier, 1975). These changes are also associated with alterations in the initial genetic programme of the host cell (Wu *et al*., 2008a).

Our results showed that the expression of the nNOS protein was down-regulated in the cytoplasm of the Nurse cell during the late stage of its formation. This phenomenon was supported also via the gene expression analyses. In light of this discovery, the development of SR, documented by other researchers (Despommier, 1975; Matsuo *et al*., 2000) appears confusing because it participates in the skeletal muscle contraction as a source of Ca^{2+}. SR is a functional deviation of the smooth endoplasmic reticulum (sER) with a specific protein composition. While the sER is involved in the synthesis of molecules, the SR stores Ca^{2+} ions and pumps them out into the sarcoplasm upon stimulation by the NO (Rossi *et al*., 2008; Percival, 2011). In light of the fact that nNOS, as a source of NO, is no longer present in the Nurse cell, and since the contractile capability of this structure is irreversibly lost, the development of the SR remains enigmatic. On one side, calcium plays a role in the calcification of the Nurse cell – an event associated with the death of the parasite (Griffin & Despommier, 2018). On the other side, calcium channel blockers were found very effective in reducing the larval count in muscles (Abdel Fadil *et al*., 2022) – a discovery that highlights the importance of host calcium ions for the nematode, and for its vital environment. In skeletal muscles, the calcium ions stored in the profiles of SR are associated with the protein calsequestrin. Upon stimulation of the RyR1by NO, $Ca^{2+}$is released into the sarcoplasm where they associate with troponin, and the muscle contraction is activated. Unregulated Ca^{2+}release may lead to pathogenic nNOS activation, causing further oxidative and nitrosative stress including RyR1 hyperactivation, which in turn provokes more Ca^{2+}leakage, leading to muscle damage, weakness, and exaggerated fatigue (Berchtold *et al*., 2000; Percival 2011). Interestingly, Dushenne and Becker muscular dystrophies are characterized by reduced nNOS expression. In addition, in dytrophin-deficient mdx skeletal muscle, the loss of nNOS expression increases muscle strength (Chang *et al*.,1996; Li *et al*., 2011) revealing the toxic activity of this enzyme, described in other pathophysiological conditions, which are also characterized by abnormal Ca^{2+} handling and oxidative stress (Hang *et al*., 1994; Ayata *et al*., 1997). It seems that NO is closely involved in all phases of the muscle damage healing process, suggesting an interrupted regulation of nNOS (Filippin *et al*., 2009). At least at the beginning of the Nurse cell development, *Trichinella* uses the same repairing process of skeletal muscle cells, induced after any trauma (Wu *et al*., 2008b) and this corroborates very well our results concerning the nNOS expression on day 14 p.i. The detected *nNOS* gene expression lags behind the level of the nNOS protein. This relates to the gene expression evaluation in the whole muscle tissue, compared to the immunohistochemical detection of the protein in individual invaded muscle cells. At a later stage, however, we could suggest that the down-regulation of nNOSin the Nurse cell of *T. spiralis* serves a protective function. This change could be a consequence of the *nNOS* gene silencing induction in the Nurse cell.

Conflict of Interest

Authors state no conflict of interest.

Authors have no potential conflict of interest pertaining to this submission to Helminthologia.

Acknowledgements

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