TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2021) 45: 873-880

doi:10.3906/vet-2101-53

In vitro investigation on extracellular traps formation of cat polymorphonuclear leucocytes against *Toxoplasma gondii*

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Received: 18.01.2021 • **Accepted/Published Online:** 06.07.2021 • **Final Version:** 26.10.2021

Abstract: Neutrophil granulocytes are one of the most important defenders of the innate immune system in the host. Moreover, neutrophils are able to reach the inflammation area and kill the pathogens using various defense strategies including neutrophil extracellular traps (NETs). However, there is still not enough information available regarding the innate immunity against *Toxoplasma gondii* in cats that are both definitive and intermediate hosts of this parasite. Therefore, we investigated the in vitro NETs formation which is induced by cat polymorphonuclear leucocytes (PMNs) against *T. gondii* tachyzoites. Firstly, PMNs were isolated from cat venous blood samples by using discontinues Percoll dilutions (72%, 63%, 54%, and 45%). Afterward, MN-tachyzoites cocultures were stained against histone (H3), neutrophil elastase (NE), and myeloperoxidase (MPO) by using monoclonal antibodies and were examined under a fluorescence microscope. The effect of different parasite doses (1:1, 1:3, and 1:5) and incubation times (30, 60, 90, and 120 min) on NETs formation was also evaluated. The presence of the extracellular DNA content was measured using a fluorometer. Confluent Vero cell cultures were used to assess the effect of NETs on the tachyzoites viability. The classical structures of NETs, such as extracellular DNA, NE, H3, and MPO were microscopically observed in the NETs formation released from cat PMNs. The amount of extracellular DNA increased in parallel with the incubation time ($p < 0.001$). The influence of the tachyzoites dose on the NETs formation was not statistically significant ($p > 0.05$). Zymosan was used as a positive control in the experiments and it was shown to be an important inducer for the NETs formation. In conclusion, as mentioned previous studies and considering our results, the NETs may be a conserved strategy to control *T. gondii* infection in hosts because of the immobilization and lethal effects.

Key words: Cat, in vitro, neutrophil extracellular traps, *Toxoplasma gondii*

1. Introduction

Toxoplasma gondii belonging to Apicomplexa phylum is an important zoonotic parasitic protozoan for several animal species as well as humans that act as intermediate hosts in the life cycle of the parasite [1]. Felidae members including domestic cats possess a significant role in the biology of *T. gondii* as both intermediate and final hosts [1]. The life cycle of *T. gondii* contains three infective stages such as tachyzoites, bradyzoites (in tissue cyst), and sporozoites (in oocyst) [1].

Tachyzoites have especially been observed in the acute phase of toxoplasmosis [2]. Tachyzoite, an aggressive form of *T. gondii*, enters into the host cell in about 26 s via the apical complex [3]. A rosette form that is also called pseudocyst occurs as a result of rapid replication of tachyzoites. When the host cell cannot support the growth of tachyzoites, cell rupture occurs and tachyzoites infect other cells [2].

During acute infection, the entrances of *T. gondii* tachyzoites into the host cell cause inflammation [2,4]. Neutrophil granulocytes which are characterized by the absence of antigen specificity and immunologic memory, as well as act the first cell in defense and immediate response to pathogens, are one of the most important cells in innate immunity [5]. Neutrophil and the other granulocytic cells (eosinophils and basophils) call polymorphonuclear granulocytes (PMNs) and neutrophils make up a major part of PMNs. The number of neutrophils in the blood circulation of cats is considerably higher than in other mammals (70%) [5].

Neutrophils have developed several defense strategies against pathogens in the organism. During inflammation, neutrophils migrate from the circulating blood to the inflammation area, and they inactivate bacteria with phagocytosis and degranulation [6]. Another defense mechanism of neutrophils is the formation of neutrophil

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extracellular traps (NETs), which is consist of chromatin fibrils and granular contents, targeting to catch and kill the pathogens in the inflammation area [7].

Extracellular trap development has been reported in some protozoa and helminths. NETs was firstly described in *Plasmodium falciparum* followed by some *L.* species *Leishmania amazonensis* [8], *L.major, L. braziliensis, L. chagasi* [9], *L. donovani* [10], *Entomoeba histolytica* [11], *Eimeria bovis* [12], *E. arloingi* [13], *E. ninakohlyakimovae*, *Besnoitia besnoiti* [14], *Cryptosporidium parvum* [15], and *Neospora caninum* [16, 17]. There are in vitro studies on NETs released from PMNs against *T. gondii* in some intermediate host [18]. This study aimed to determine the in vitro NETs formation in cat PMNs against *T. gondii* tachyzoites.

2. Material and methods

2.1. Neutrophil isolation from venous blood samples

All animal experiment procedures were reviewed and approved by the Ethics Commission of Kırıkkale University (02.02.2016, no: 16/01). Cat neutrophil isolation was carried out according to method described by Wanderley et al. (2014) with minor modification [19]. Briefly, Percoll solution (Sigma) was prepared at four different concentrations (72%, 63%, 54%, and 45%) using sterile Hank's balanced salt solution (HBSS, Sigma). The venous blood samples were collected from *Vena cephalica* of adult cats in good health (n = 5) into anticoagulant tubes. After mixed with sterile 0.02% PBS-EDTA (equal volumes), the blood samples were layered into sterile polystyrene tubes (Falcon) with four different concentrations of Percoll solutions (Sigma). After centrifugation (500 × g, 22 °C, 35 min) (Thermo Scientific SL 16R), the cell layer between 72% and 63% intensely Percoll layers were collected using a sterile pipette. HBSS (1x) was added into the cells and centrifuged (300 × g, 4 °C, 10 min). Finally, the pellet was diluted using RPMI-1640 (Sigma). Cat PMNs was counted via Neubauer chamber and diluted with RPMI 1640 as 1 × 10^5/100 µL. After Diff-Quick staining neutrophil purity in cat PMNs was microscopically examined (Bio Optica, Italy). The viability of PMNs was evaluated using the trypan blue dye.

2.2. Fluorometric analysis of NETs structures

PMNs and tachyzoites were placed into sterile tubes with different doses (1:1, 1:3, and 1:5) and incubated at different times (30, 60, 90, and 120 min) (5% CO_2, 37 °C) to determine the effect of different parasite doses on NETs development. After incubation time, micrococcal nuclease (5 U, NEB) was added to the cat PMN-tachyzoites cocultures and the mixtures were incubated (5% CO_2, 37 °C, 15 min) and then were centrifuged (300 × g, 5 min). The supernatants were dropped into flat-bottom 96-well plates (Nunc, Sigma-Aldrich), each supernatant was worked in duplicate. Picogreen extracellular DNA Dye (5 µM, Invitrogen) was added to the wells and incubated at room temperature for 15 min in dark condition. PMNs induced by Zymosan (20 nM Invitrogen) was used as positive control while untreated PMNs were used as a negative control in the experiments. The fluorescence level was measured using a fluorometer (485 nm excitation/538 nm emission) (Fluoroskan Ascent FL, Thermo Scientific). These experiments were repeated three different times and the arithmetic mean of the results was considered.

2.3. Fluorescence microscopic analysis of NETs structures

Tachyzoites were labelled using CellTrace CFSE Cell Proliferation Kit (5 µM, Invitrogen) was used to label and prove the visibility of tachyzoites within NETs structures. Briefly, tachyzoites and CFSE were incubated at 37 °C for 10 min in the tube. Afterward, cold PBS including 10% FCS was added. The labelled tachyzoites were triple washed with PBS (400 × g, 10 min) and protected from light. Cat PMNs and labelled tachyzoites (1:3) were placed on coverslips coated with poly-L-lysine and incubated for 1 h (37 °C, 5% CO_2). Then, paraformaldehyde (4%) and Triton X-100 (0.1%) were added. After the reaction was blocked with bovine serum albumin (1%), the samples were incubated with some monoclonal antibodies [anti-NE; 1:1000, sc-55548, Santa Cruz, anti-MPO; 1:1000, sc-52707, Santa Cruz, and antihistone (H3); 1:1000, sc-374669, Santa Cruz] (1 h, 37 °C, 5% CO2) to detect of NE, MPO, and histone (H3) in NETs structure. Secondary antibodies (FITC labelled IgG2b; 1:100, ABIN 637988 for histone (H3), FITC labelled IgG1, 1:100, sc358546, Santa Cruz for NE and MPO were used in the experiments. Finally, the samples were stained with Sytox Orange extracellular dye (Invitrogen) (1:30,000) at room temperature for 5 min in dark condition).

After placed one drop of Mowiol, the coverslips were cautiously put onto slides and visualized using fluorescence microscope (Leica DM IL Led, 470 nm excitation/515 nm emission). ImageJ (version 2.0.0-rc-43/1.50 g) was used to generate composite images. Briefly, the obtained images were merged using channel merge function including different filters (red and green) of ImageJ Program (version 2.0.0-rc-43/1.50g).

2.4. Vero cell culture assay

Vero cell culture was used as a host cell in vitro, to determine the effect of NETs on the tachyzoites viability (entry into host cells). Cat PMN's and *T. gondii* tachyzoites cocultures (1:3) were taken into sterile tubes (samples were studied in duplicate). The tubes were incubated for 3 h in an incubator at 37 °C, 5% CO 2 (NUVE MN120). To determine whether the NETs formation has only mechanically immobilized the tachyzoites or has a lethal effect on the tachyzoites, DNase I (90 U, Sigma) was added into some tubes 15

min before the end of the 3-h incubation. The contents of tubes (with and without DNase I) were seeded on wells of 6-cell culture plates coated with Vero cells (after 80% confluency-after 24 h of passage). Each sample was studied in duplicate. To allow the tachyzoites to enter into cells, the plates were incubated for 2 h in an incubator (37 °C, 5% CO_2). Then, the plates were washed twice with sterile PBS to remove any tachyzoites that did not enter the cell. After adding DMEM to the plates, the samples were incubated for 22 h under the same conditions. As a control, untreated tachyzoites were seeded into the wells coated with Vero cells. All wells were incubated for 22 h (37 °C, 5% CO_2). Finally, the infected cells were counted in ten randomly selected microscopic areas and determined the percentage of them (Olympus CKX41 inverted microscope).

2.5. Obtaining T. gondii tachyzoites

Tachyzoites (RH strain) were obtained from peritoneal fluid of experimentally infected *Swiss albino* mice after 48 h of infection. This fluid was centrifuged with sterile saline solution (pH 7.2) to purify the tachyzoites (1000 × g for 10 min). The tachyzoite numbers were calculated using Neubauer chamber. The tachyzoite dilutions were prepared using the RPMI-1640 solution.

2.6. Statistical analysis

The descriptive statistics of the variables are shown as arithmetic mean ± standard error. Before the significance tests, Shapiro–Wilks test was examined for the normality of the parametric test assumptions and Levene's test for the homogeneity of the variances. The general linear model for repeated measures was used to examine the changes in time series of different dose groups in terms of AU measurements. In the model, the terms of between-subject factor, within-subject factor and dose × time interaction are included. The Huynh–Feldt correction was applied since the assumption of sphericity was not achieved when the model was constructed. The Tukey test was used as an advanced stage test for significant effects. For all statistical analyses SPSS 14.01 package program. p value less than 0.05 ($p < 0.05$) was considered to be statistically significant.

3. Results

3.1. Quantitative analysis of NETs against *T. gondii* tachyzoites

The amount of extracellular DNA increased in parallel with the incubation time in the cat PMN-tachyzoites cocultures ($p < 0.001$) (Figure 1). There was not found any relationship between the amount of extracellular DNA and the tachyzoite concentrations ($p > 0.05$) (Figure 2). Zymosan (20 nM) used as a positive control was found to be a good trigger for extracellular trap formation in cat PMNs.

3.2. Observing classical features of NETs structures against *T. gondii*

In this study, extracellular DNA, histone, NE, and MPO, which is the classical structure of NETs, have been demonstrated in the extracellular traps released from the cat PMNs after encounters with *T. gondii* tachyzoites. At the beginning of NETs reaction, nucleus structure and its lobular form disappeared in the neutrophils. Then, the trap-like filaments were released from the neutrophils to the extracellular space after the ruptured cell membrane. DNA, backbone structure of NETs, was observed in the extracellular area after stained with Sytox Orange dye (Figures 3A, 3D, and 3G). NE, MPO, and histone (H3) were detected on extracellular DNA filaments (Figures 3B, 3E, and 3H). Labelled *T. gondii* tachyzoites were observed in NETs as entrapped (Figures 3C, 3F, and 3I).

3.3. Vero cell culture assay

The number of tachyzoites entered into Vero cells in the cat PMN-tachyzoites cocultures (1:3 ratio) were detected higher compared to the control group at the finished of incubation. It was determined that the number of infected Vero cells increased with DNase I addition which disrupted the extracellular traps and tachyzoites were released (Figure 4). The number of tachyzoites reached generally 16 as a result of replication in the same cell at the 22nd h of incubation (in both groups: untreated and treated with DNase I PMN-tachyzoites cocultures, 1:3 ratio) while the number of tachyzoites was commonly determined as 8 in the control group (Figure 5).

4. Discussion

Recently, extracellular traps released from PMNs have been considered as a powerful weapon against bacteria, viruses, fungi, and parasites in the defense of innate immunity [20]. Production of NETs in response to some pathogens has been defined for various animal species including mice, cattle, horse, chicken, dog, sheep, and fish [7, 21–27]. *Toxoplasma gondii* is NETs inducer from neutrophils of different animals and humans [18, 27]. However, the role of PMNs in cat toxoplasmosis still remains poorly explored. In this study, it was demonstrated that the extracellular traps released from the cat PMNs to *T. gondii* tachyzoites in vitro and this part was presented as the first report at a scientific congress [28]. Firstly, the classical structures of NETs, the extracellular DNA, NE, MPO, and histon (H3) were observed using fluorescence microscope in cat PMNs against *T. gondii* tachyzoites. Afterward, time and dose-dependent changes of NETs were also evaluated.

There are some in vitro studies to determine the amount of extracellular DNA released from PMNs against some parasites depending on the incubation time [10,13,15,29]. Although some researchers have revealed that the amount of extracellular DNA does not depend on the PMNs-

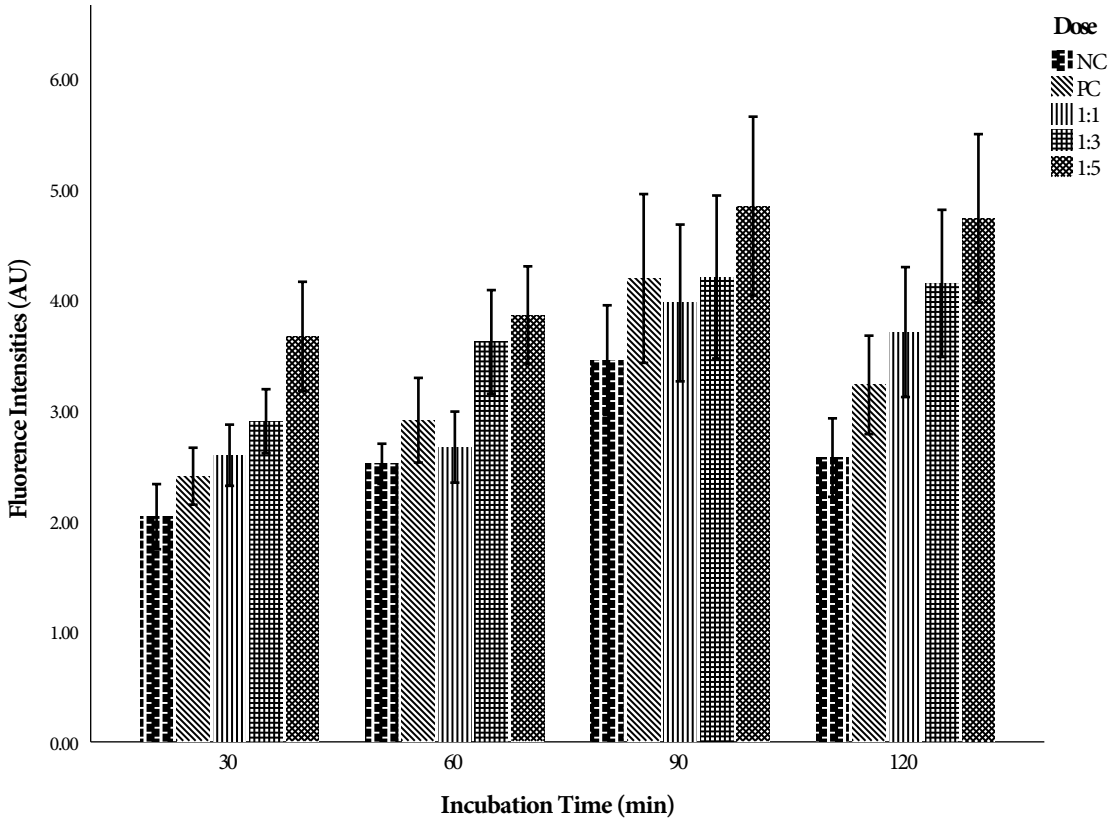

Figure 1. The amount of extracellular DNA released from cat polymorphonuclear leucocytes (PMNs) – *T. gondii* tachyzoites cocultures (1:1, 1:3 and 1:5) depending on the different incubation times (30, 60, 90, and 120). Zymosan-induced PMNs were used as positive control (PC) and only PMNs were used as negative controls (NC). (AU: arbitrary unit).

parasite incubation time, it was commonly reported that the amount of DNA released from human, goat, bovine, and canine PMNs increased parallel with incubation time against some parasites such as *L. donovani* GFP promastigotes [10], *E. arlongi* sporozoites [13], *C. parvum* sporozoites [15], and *N. caninum* tachyzoites [30], respectively. Similar to our results, it has been emphasized that the increase in the amount of extracellular DNA released from different hosts PMNs (mouse, human, harbour seal, sheep, and cattle) against *T. gondii* tachyzoites is also time-dependent in various studies [18,27,31]. We also showed that the time-dependent increase was statistically significant ($p < 0.001$).

Previously, the effect of parasite concentration on the amount of extracellular DNA released from PMNs against some parasites has also been studied. It was reported that there is an enhancing effect of the parasite dose on the amount of the DNA released from human, bovine, and dog PMNs against different parasites such as *L. donovani* [10], *L. major* [10], *E. bovis* [12], *B. besnoiti* [14], and *N. caninum* [17], respectively. However, similar to our results, it was demonstrated that parasite doses had no effect on the amount of DNA released from different hosts such as harbour seal, cattle, and sheep PMNs against *T. gondii* tachyzoites [27,31]. This result has also been reported in goat PMNs against *N. caninum* tachyzoites. We showed that the dose-dependent difference of the amount of extracellular DNA from cat PMNs was not statistically significant ($p > 0.05$).

NETs structure can immobilize and kill pathogens [32]. NETs structure contains histone, which has an antimicrobial effect, and also MPO and NE, all of which contribute to killing the pathogens [32]. There are some studies on the lethal effects of NETs from human PMNs against *L. amazonensis* promastigotes and amastigotes [8], *L. major* [10], and *L. chagasi* promastigotes because of the presence of histone (9). On the other hand, it was shown that the 3'-nucleotides/nuclease enzyme allows *Leishmania* parasites to escape killing by NETs [33]. In addition, the immobilization effect of NETs from bovine PMNs against *E. bovis* sporozoites [12], *B. besnoiti* tachyzoites [14], and *C. parvum* sporozoites [15] has been reported. Considering the toxoplasmosis, immobilization and lethal effects have been determined in the different host (mouse, human, cattle, and sheep) PMNs [18, 27]. The immobilization effect of

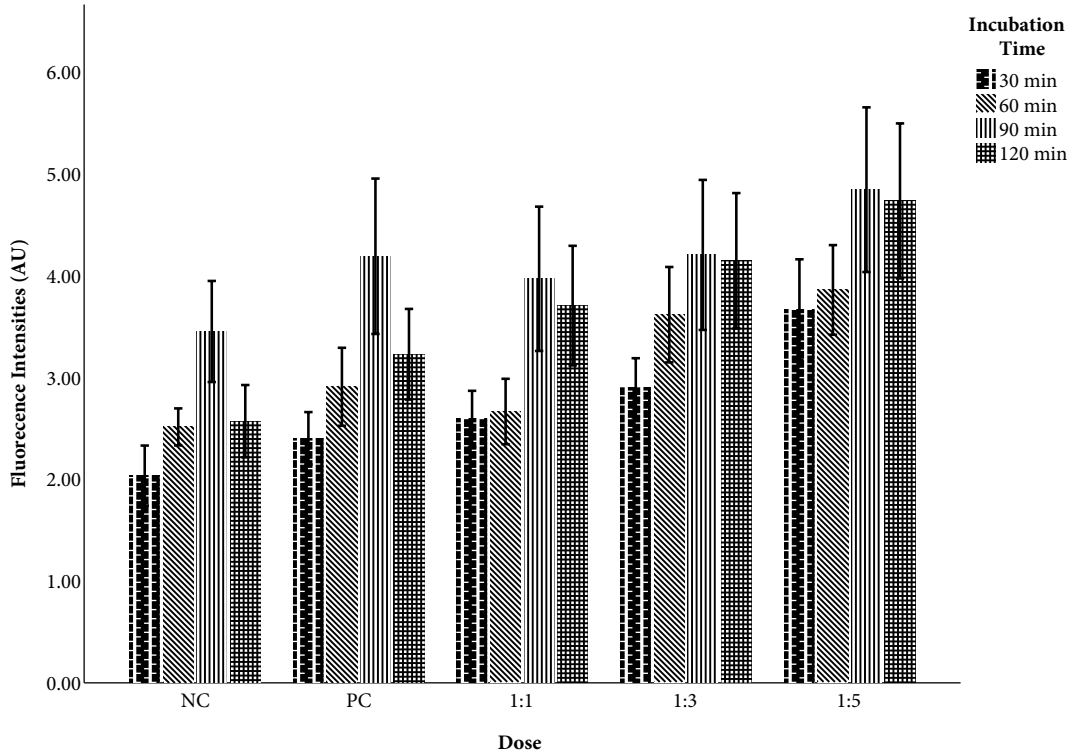

Figure 2. The amount of extracellular DNA released from cat polymorphonuclear leucocytes (PMNs) – *T. gondii* tachyzoites cocultures (1:1, 1:3 and 1:5) depending on the different doses. Zymosan-induced PMNs were used as positive control (PC) and only PMNs were used as negative controls (NC). (AU: arbitrary unit).

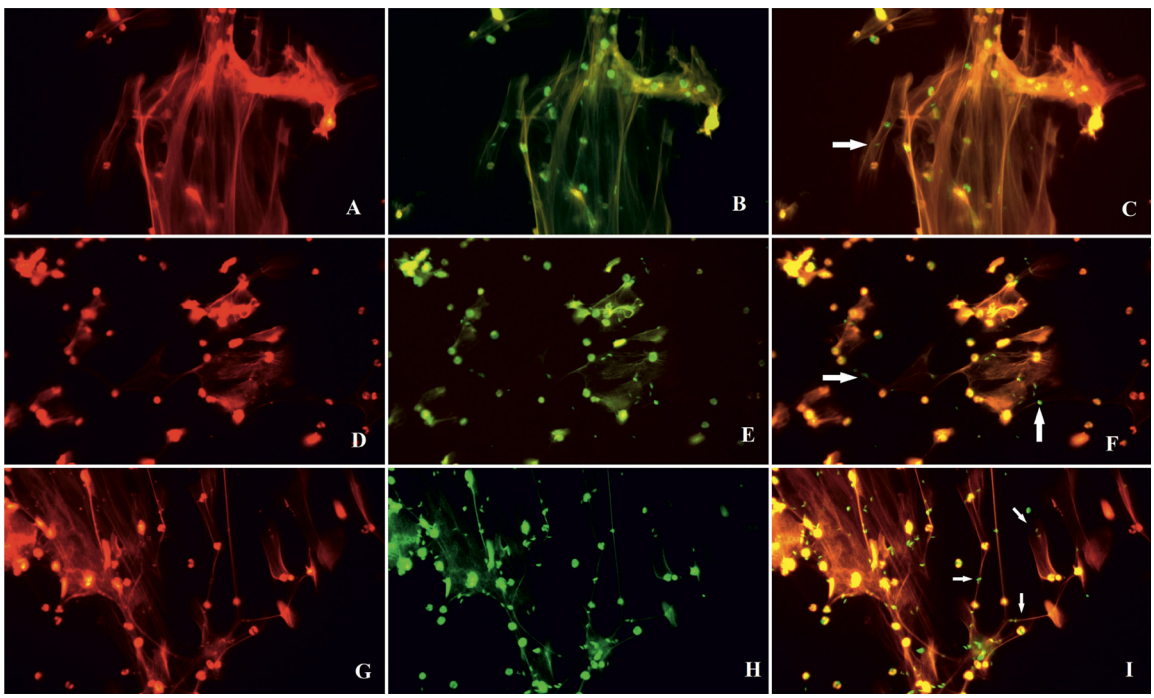

Figure 3. Visualization of the classic features of the extracellular traps released from the cat's neutrophils after incubated with *T. gondii* tachyzoites for 1 h; A, D, G: the detection of extracellular DNA with Sytox Orange dye; B, E, H: detection of histone (H3) (fluorescence microscope Leica DM IL Led, 470 nm excitation/515 nm emission); C, F, I: merged of the two previous images (ImageJ software, version 2.0.0-rc-43/1.50g).

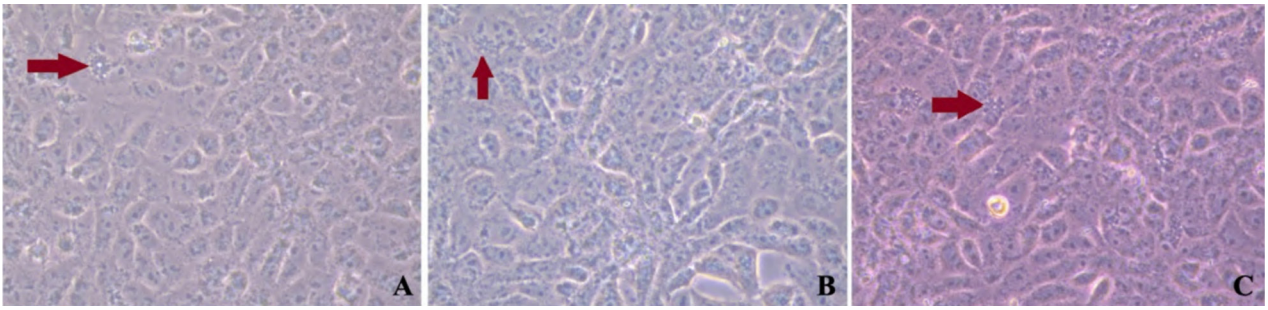

Figure 4. Vero cells were used as host cell in the culture assay; A) PMN: tachyzoites (1:3); proliferating 16 replicate tachyzoites; B) 1:3 group with DNase; 16 replicate tachyzoites; C) Control with tachyzoites: RPMI (1:3); 16 replicate tachyzoites; tachyzoites that enter the cell are indicated by red arrows (Olympus CKX41).

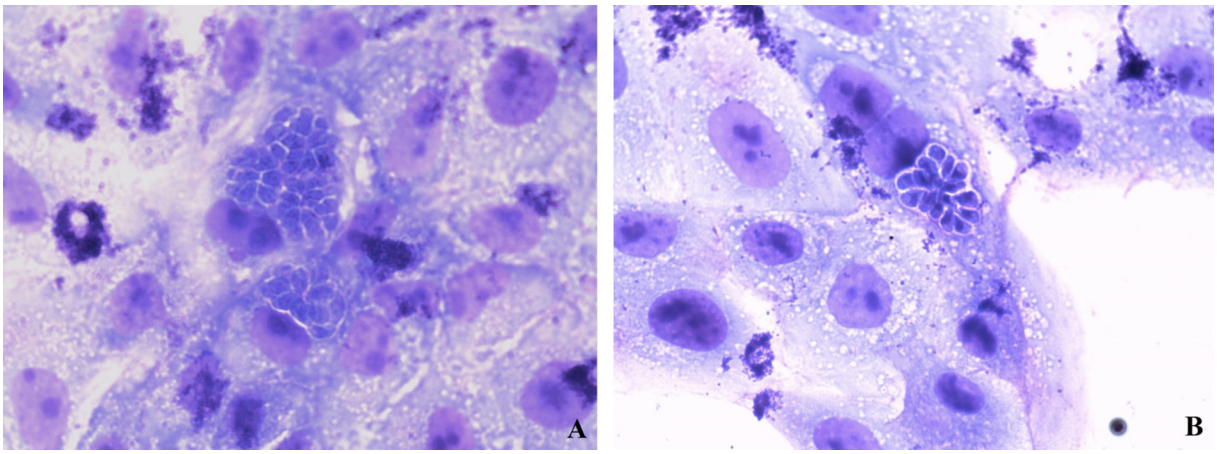

Figure 5. Host cell assay with Vero cells; with (A) and without DNase treatment (B); Giemsa staining (Leica DM750, Objective × 100).

NETs structures released from cat PMNs against *T. gondii* tachyzoites was detected in our in vitro study. Currently, the lethal effect of NETs structure was firstly revealed by Macedo et al. [34].

Lastly, some chemical substances such as zymosan are known to be NETs inducers from neutrophils [35]. Zymosan activity for some animals PMNs (cattle, goats, humans, dogs, and harbor seal) was previously evidenced [10,13,16,30]. Therefore, zymosan is used as positive controls in vitro NETs experiments [10, 13–16]. In the present study, zymosan was also found to be a good extracellular trap trigger for cat PMNs.

In conclusion, we showed the NETs formation, which may act an important role in defense mechanism in hosts, in cat PMNs against *T. gondii* tachyzoites in vitro. The classical structure of NETs such as histone (H3), MPO, NE, and extracellular DNA microscopically was observed from cat PMNs. Similar to previous studies, the amount of extracellular DNA increased in parallel with the PMN-tachyzoites cocultures incubation time. Zymosan, which is used as a positive control in this study, has been shown as an important inducer for NETs in cat PMNs. Future studies are needed to clarify which receptors *T. gondii* tachyzoites bind to in cat neutrophils, determine the differences in these receptors in cats and other hosts, and define the granular content of cat neutrophils.

Acknowledgment

Toxoplasma gondii RH tachyzoites were kindly obtained from Dr. Cahit Babur (The Public Health Institution of Turkey). The authors thank Dr. Doğukan Ozen (Department of Biostatistics, Faculty of Veterinary Medicine, Ankara University) for statistical analysis. This study is produced from PhD dissertation. Some part of this work was presented at the 5th European Immunology & Innate Immunity, July 21-23, 2016 Berlin, Germany.

Ethical statement

This study was approved by the Kırıkkale University Animal Experiments Local Ethics Committee (02.02.2016, no: 16/01).

Conflict of interest

The authors declared that there is no conflict of interest.

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