Turkish Journal of Veterinary & Animal Sciences

Volume 44 | Number 4 Article 17

1-1-2020

In vitro investigation of NETosis reaction developing from dog polymorphonuclearneutrophils to Toxoplasma gondii

GÜNEŞ KARAKURT

KADER YILDIZ

TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

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

Research Article

Turk J Vet Anim Sci
(2020) 44: 886-893

doi:10.3906/vet-2002-6

In vitro investigation of NETosis reaction developing from dog polymorphonuclear neutrophils to *Toxoplasma gondii*

Güneş KARAKURT[1,*], **Kader YILDIZ**[2]

[1]Department of Parasitology, Graduate School of Health Science, Kırıkkale University, Kırıkkale, Turkey
[2]Department of Parasitology, Faculty of Veterinary Medicine, Kırıkkale University, Kırıkkale, Turkey

Received: 04.02.2020 • **Accepted/Published Online:** 14.06.2020 • **Final Version:** 18.08.2020

Abstract: *Toxoplasma gondii* is known to develop extracellular traps from neutrophils in some animal species such as mice, cattle, sheep, cats, and donkeys. This study aimed to investigate the extracellular trap structures formed in dog polymorphonuclear leukocytes (PMNs) to *T. gondii* tachyzoites in vitro. Dog PMN was isolated using Percoll dilutions (45%, 54%, 63%, and 72%). After incubation with tachyzoites, the extracellular traps originating from the dog PMNs were observed in the extracellular areas. Histones (H3), myeloperoxidase (MPO), and neutrophil elastase (NE), the characteristic features of NETosis reaction, were detected in extracellular areas. The tachyzoites were observed between the extracellular trap structures. A positive correlation was detected between the parasite concentration and extracellular traps formation but they were not statistically significant ($P > 0.05$). Time-dependent relationships were also not statistically significant ($P > 0.05$). The extracellular traps released in the PMN–tachyzoite culture increased until the 60th min of incubation. Reactive oxygen species, MPO, and NE activities were observed in the PMN–tachyzoite culture during the incubation period. The development of extracellular traps against *T. gondii* in dog PMNs is reported for the first time in this study. However, it could not be determined whether the extracellular traps released from dog PMNs only mechanically immobilize or have some lethal effect on tachyzoites.

Key words: In vitro, dog, NETosis, polymorphonuclear leukocyte, *Toxoplasma gondii*

1. Introduction

Three infectious stages of *Toxoplasma gondii* are known: tachyzoite, bradyzoite (in tissue cyst), and sporozoite (in oocyst) [1]. The final hosts of the parasite are domestic cats and other members of the Felidae family. Intermediate hosts consist of humans and different animal species including dogs [1]. *T. gondii* infections can be observed as neuromuscular (ataxia), respiratory and gastrointestinal symptoms (diarrhoea), or a generalized infection in dogs [2,3,4,5].

Neutrophils are the most common innate immunity cells in humans and animals. These cells fight pathogens entering the body using different mechanisms such as phagocytosis and degranulation, as well as NETosis [6]. When a neutrophil encounters pathogens or certain molecules in the organism, the nuclear and granular contents are mixed with each other in the neutrophil. Then, extracellular traps (NETs), including antimicrobial enzymes such as myeloperoxidase (MPO) and neutrophil elastase (NE), are released to the extracellular area [6,7]. Extracellular traps have been reported to form against many bacteria, fungi, and viruses. Some protozoa [8–17] and helminths [18–23] have been reported to trigger the formation of NETs. Not only live parasites but also parasitic lysates or heat-inactivated parasites have been reported to develop extracellular traps from polymorphonuclear leukocytes (PMNs) [8].

Toxoplasma gondii develops the extracellular traps from PMNs of some animal species including cattle, sheep, cat, donkey, mouse, harbour seal, and dolphin [16,24–28]. In this study, we aimed to investigate the extracellular trap structures formed in dog PMNs after being confronted with *T. gondii* tachyzoites in vitro. This study also aimed to determine the effect of parasite concentration and incubation time on the formation of extracellular traps. Also, reactive oxygen species (ROS), MPO, and NE activities were determined in extracellular traps formed against *T. gondii.*

2. Materials and methods

The Ethics Committee decision for all animal manipulations was received from the Animal Experiments

* Correspondence: gunes_karakurt@hotmail.com

Local Ethics Committee of Kırıkkale University (Meeting date: 06.09.2017; decision no: 17/32).

2.1. Isolation of PMNs

Five mL blood samples were collected from the *Vena cephalica* of five clinically healthy dogs in anticoagulant tubes containing EDTA. PMNs were isolated from the venous blood samples as described by Sursal et al. [29]. Percoll (Merck KGaA, Darmstadt, Germany) dilutions (45%, 54%, 63%, and 72%) were used for the isolation of PMN. The PMNs were counted using a Neubauer counting chamber. To determine the viability of PMNs, 0.4% Trypan blue (Merck KGaA, Darmstadt, Germany) staining was performed. The neutrophil rate was determined in the isolated PMN using the Diff-Quick dye solution (Bio Optica, Italy).

2.2. *Toxoplasma gondii* tachyzoites

Toxoplasma gondii is passed from mice in the Parasitology Laboratory of the Turkish Public Health Institution, Ankara for use in the Sabin Feldman Dye Test. Tachyzoites were obtained from an experimentally infected mouse peritoneum via peritoneal lavage. The tachyzoite numbers were counted with light microscopy (Leica DM750) using Neubauer's chamber. Then, the tachyzoite suspension was diluted as 1×10^5/100 µL using RPMI-1640.

2.3. Quantitative analysis of extracellular DNA released in NETosis reaction

PMNs and tachyzoites (1:1, 1:2, and 1:3 concentrations) were placed in separate sterile reaction tubes. Phorbol 12-myristate 13-acetate (PMA) (Merck KGaA, Darmstadt, Germany, 50 nM) stimulated canine neutrophils were used as positive controls. The tubes (experiment and control) were kept in the CO_2 incubator (Nuve MN120) set at 37 °C for 30, 60, and 120 min (5% CO_2). At the end of this period, micrococcal nuclease (NEB, 5 U) was added into the tubes and the incubation was continued for 15 min under the same conditions. Following centrifugation, the supernatant was placed in black microtitration plate wells and Sytox Green extracellular DNA stain (Thermo Fisher Scientific, Waltham, MA, USA, 5 µM) was added. The plate was kept in the dark for 15 min and measured using a fluorometer (Fluoroscan Ascent FL, Thermo Scientific) (460 nm excitation/538 nm emission).

2.4. Immunohistochemical staining of extracellular traps

For better monitoring the tachyzoites among the extracellular traps formed during the NETosis reaction, the tachyzoites were stained with the permeable fluorescent dye 5- (and -6)-carboxyfluorescein diacetate N-succinimidyl ester (CellTrace CFSE Cell Proliferation Kit, Thermo Fisher Scientific, Waltham, MA, USA, 5 µM) as recommended by the manufacturer. The dog PMNs (1×10^5/100 µL) and fluorescence-labelled tachyzoites were placed in a 1:1 concentration on poly-L-lysine coated coverslips and kept in the incubator for 1 h (37 °C, 5% CO_2). Subsequently, 4% paraformaldehyde and 0.5% Triton X-100 were added. Histone (H3), MPO, and NE, which are characteristic of NETosis, were stained with appropriate primary and secondary antibodies (Table). Then, extracellular DNA was stained with Sytox orange dye for 5 min (1:30,000). The fluorescence was examined under a fluorescence microscope (Leica IL Led Fluo) (495 nm excitation/520 nm emission).

2.5. Measurement of ROS, MPO, and NE activities during NETosis reaction

The dog PMNs and tachyzoites (1:2) were placed on a black microtitration plate. Dog PMNs treated with PMA (100 nM) were used as a positive control. At the beginning of the incubation, 2',7'-dichlorofluorescin diacetate (Merck KGaA, Darmstadt, Germany, 10 µg/mL) and MeoSuc-Ala-Ala-Pro-Val-chloromethyl-ketone (Merck KGaA, Darmstadt, Germany, 3 mg/mL) were added to the wells to determine ROS and NE activity, respectively. For the determination of MPO activity, Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was applied as recommended by the manufacturer. All plates were placed in a fluorometer set at 37 °C. The measurements were obtained at intervals of 10 min during the incubation (for 150 min) (485 nm excitation/520 nm emission).

2.6. Statistical analysis

Dose and time-dependent results of this study were statistically analysed using SPSS 22 (SPSS Inc., Chicago, IL, USA). All hypothesis tests were performed with a confidence level of 95%. Descriptive statistics were used to calculate the arithmetic mean and standard error. Firstly, a one-way analysis of variance (ANOVA) was performed for paired comparisons, and then the LSD test was used to determine whether there was a difference in paired comparisons. The normality hypothesis required for the realization of ANOVA was tested by Shapiro-Wilk, and the first type error, where the data subject to binary comparisons were normally distributed, was accepted as 0.05. At the same time, a homogeneous variance hypothesis, which is the second condition for ANOVA, was tested by Leneve, and a sufficient P value was reached at 0.05 error level.

3. Results

The ratio of neutrophils in the isolated PMNs was found to be 92%. Following Trypan blue staining, 98% of the isolated neutrophils were detected as viable. After coming in contact with tachyzoite, a NETosis reaction was observed in the dog PMNs (Figures 1–3). The extracellular traps have been identified as in the cloud shape (Figure 1a). These extracellular structures had the property of DNA (Figure 1b). Histones (H3) were observed on the extracellular trap

Table. Primary and secondary antibodies used in immunohistochemical staining

Primer antikor	Dilution	Sekonder antibody	Dilution
Anti - histon (H3) monoclonal antibody	1 : 1000	FITC marked IgG_{2b}	1 : 100
Anti - MPO monoclonal antibody	1 : 1000	FITC marked IgG_1	1 : 100
Anti - NE monoclonal antibody	1 : 1000	FITC marked IgG_1	1 : 100

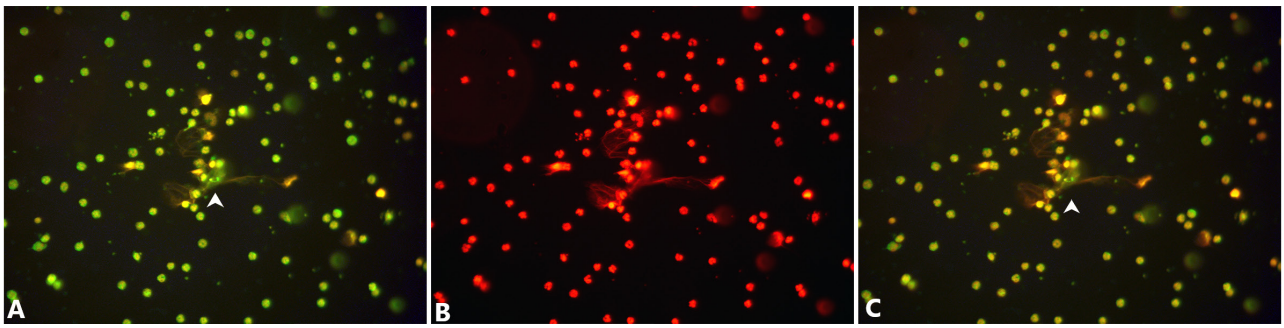

Figure 1. Fluorescence micrograph of extracellular trap structures triggered by *Toxoplasma gondii* tachyzoites. After staining with 5-(and -6)-carboxyfluorescein diacetate *N*-succinimidyl ester (5 µM), tachyzoites were incubated for 1 h with dog polymorphonuclear neutrophils (1:1). Then the culture was probed for histones (H3) (A) using antihistone antibody and stained with Sytox orange dye for extracellular DNA backbone (B). A composite image (C) was generated using Image J (version 2.0.0 – rc – 43/1.50 g). Tachyzoites to be entrapped in NET structures (arrows) (× 40).

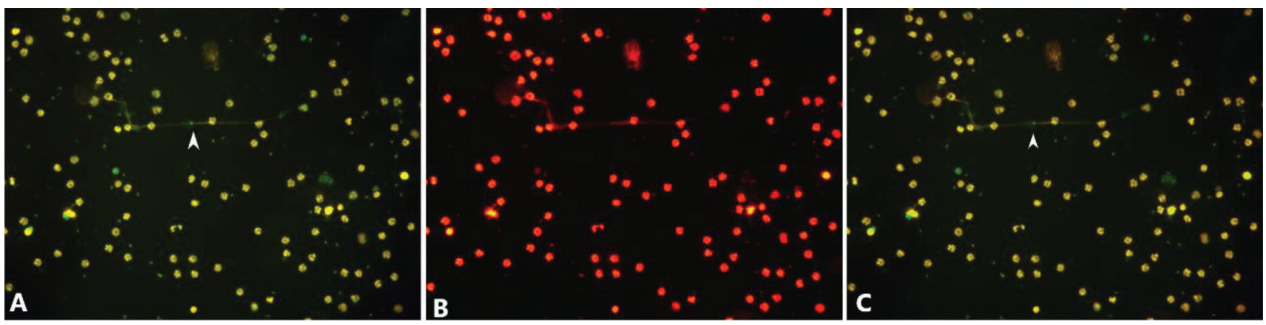

Figure 2. Fluorescence micrograph of extracellular trap structures triggered by *Toxoplasma gondii* tachyzoites. After staining with 5-(and -6)-carboxyfluorescein diacetate *N*-succinimidyl ester (5 µM) tachyzoites were incubated for 1 h with dog polymorphonuclear neutrophils (1:1). Then the culture was probed for myeloperoxidase (A) using antiMPO antibody and stained with Sytox orange dye for extracellular DNA backbone (B). A composite image (C) was generated using Image J (version 2.0.0 – rc – 43/1.50 g). Tachyzoites to be entrapped in NET structures (arrows) (× 40).

structures after they were stained with adequate antibodies (Figure 1c). The extracellular traps have been identified as in the strand shape (Figure 2a). These extracellular structures had the property of DNA (Figure 2b). MPO was observed on the extracellular trap structures after staining with adequate antibodies (Figure 2c). The extracellular traps have been identified (Figure 3a). These extracellular structures had the property of DNA (Figure 3b). NE was observed on the extracellular trap structures after staining with adequate antibodies (Figure 3c). The fluorescence-labelled tachyzoites are observed between the NETs. The structures of the extracellular trap appear to adhere to the tachyzoites and immobilize them.

3.1. Variation of extracellular DNA based on tachyzoite concentration and incubation time

A positive relationship was detected between the parasite concentrations and extracellular DNA amounts. However, the differences were not statistically significant ($P > 0.05$). Figure 4 shows a time-dependent variation of the extracellular traps formed in the culture of PMN-tachyzoites. It was determined that the extracellular DNA amount increased up to 60 min of incubation in all experiments and control groups. However, the differences were not statistically significant ($P > 0.05$). PMA used as a positive control was found to be a good activator of NETosis for canine neutrophils.

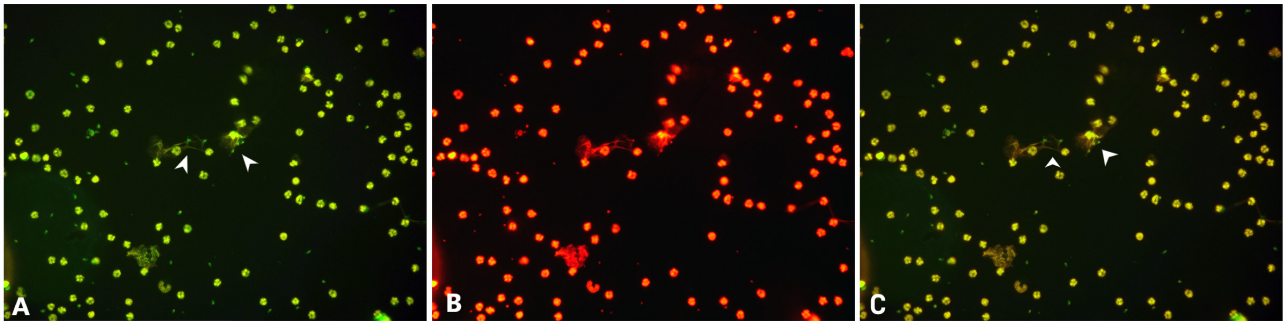

Figure 3. Fluorescence micrograph of extracellular trap structures triggered by *Toxoplasma gondii* tachyzoites. After staining with 5-(and -6)-carboxyfluorescein diacetate *N*-succinimidyl ester (5 µM) tachyzoites were incubated for 1 h with dog polymorphonuclear neutrophils (1:1). Then the culture was probed for neutrophil elastase (A) using antiNE antibody and stained with Sytox orange dye for extracellular DNA backbone (B). Composite images (C) were generated using Image J (version 2.0.0 – rc – 43/1.50 g). Tachyzoites to be entrapped in NET structures (arrows) (× 40).

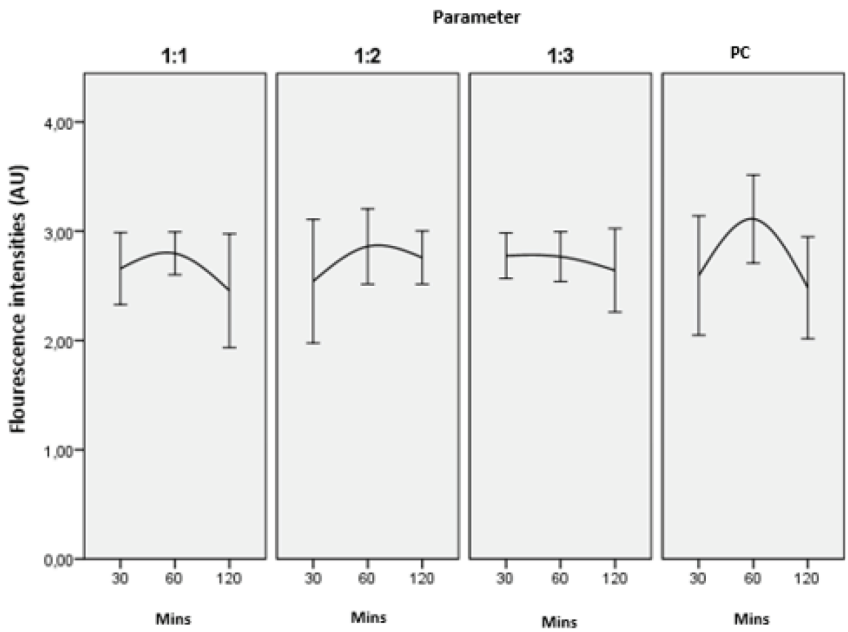

Figure 4. Time-dependent alteration of extracellular DNA amounts in the dog polymorphonuclear neutrophils-tachyzoite cultures (1:1, 1:2, 1:3) (PC: positive control) (AU: arbitrary unit) ($P > 0.05$).

3.2. Determination of ROS, MPO, and NE activity during incubation

During the incubation, a time-dependent increase of ROS was detected in both the PMN-tachyzoite cultures and positive control. The level of ROS was higher in the positive control group than that of PMN-tachyzoites cultures during incubation (Figure 5).

MPO activity is shown in Figure 6. The MPO activity showed a time-dependent increase both in the PMN-tachyzoite culture and in the positive control during incubation. In both experiments and positive control groups, MPO levels tended to increase during the first 40 min of incubation. After this point, it remained constant for 70 min and then tended to decrease slowly. The amount of MPO in the positive control was higher than in the PMN-tachyzoite culture.

NE activity is shown in Figure 7. Both the PMN–tachyzoite culture and the positive control yielded a time-dependent increase in the amount of NE released. The NE activity was higher in the positive control group than that of the tachyzoite-induced reaction.

4. Discussion

NETosis is known to occur in dog PMNs [30]. The NETosis reaction has been reported against some parasites such as *Neospora caninum* and *Dirofilaria immitis* in dog PMN

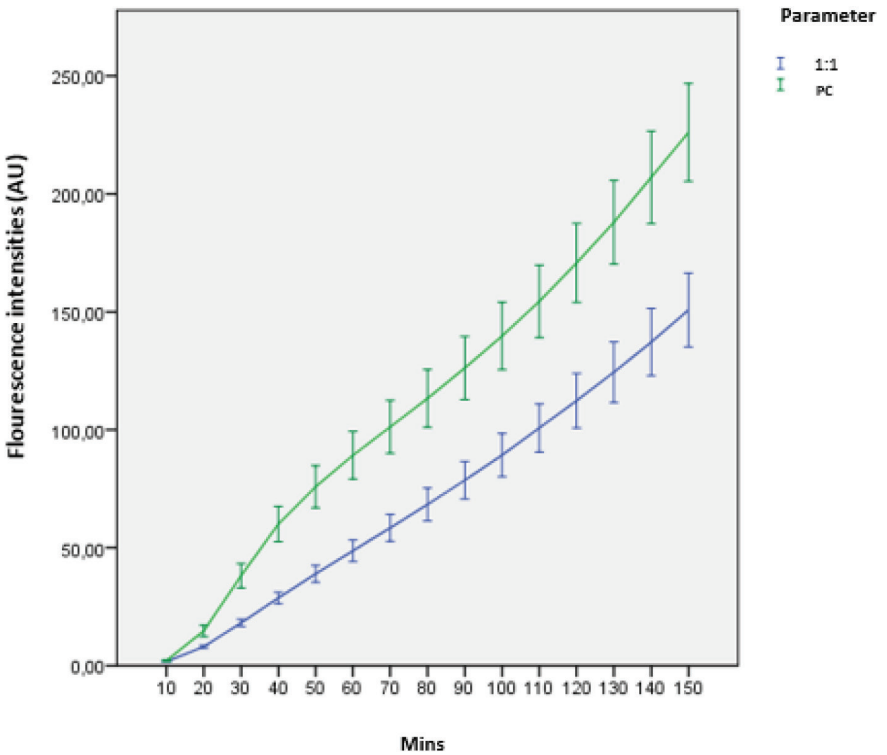

Figure 5. Time-dependent alteration of reactive oxygen species (ROS) activity in the dog polymorphonuclear neutrophils (PMN)-tachyzoite cultures (1:1). ROS activity was measured in a fluorometer (485 nm excitation/538 nm emission). Phorbol 12-myristate 13-acetate-induced PMNs were used as the positive control (PC) (AU: arbitrary unit).

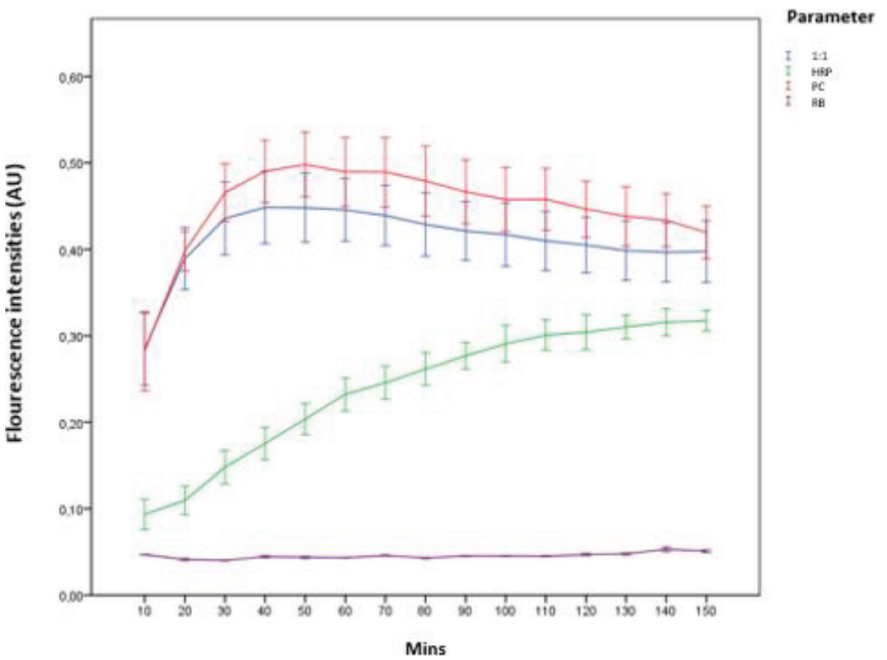

Figure 6. Time-dependent alteration of myeloperoxidase activity in the dog polymorphonuclear neutrophils (PMN)-tachyzoite cultures (1:1). Neutrophil elastase activity was measured in a fluorometer (485 nm excitation/538 nm emission). Phorbol 12-myristate 13-acetate-induced PMNs were used as positive control (PC). (HRP: horseradish peroxidase; RB: reaction buffer; AU: arbitrary unit).

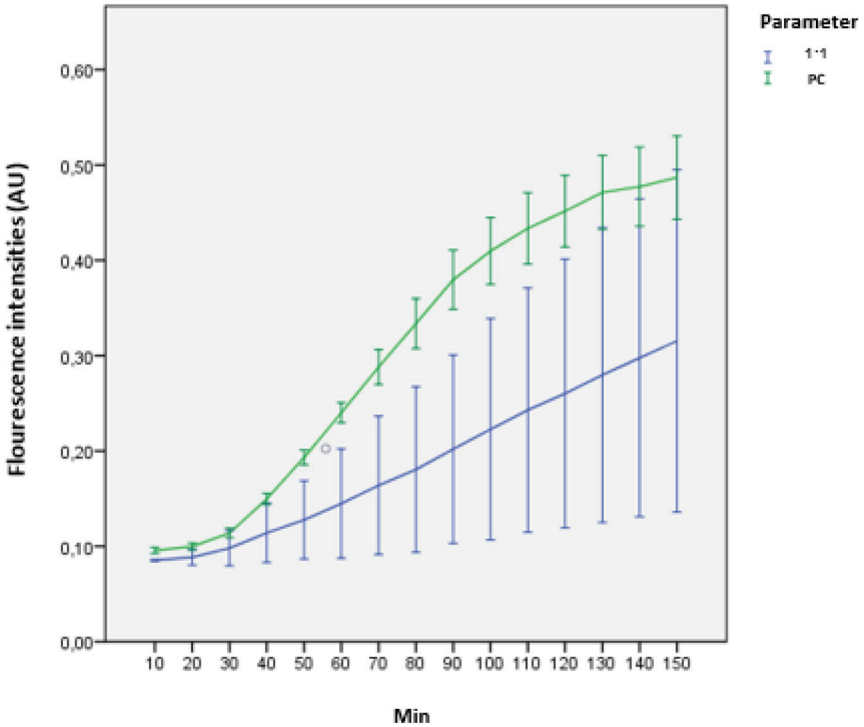

Figure 7. Time-dependent alteration of neutrophil elastase activity in the dog polymorphonuclear neutrophils (PMN)-tachyzoite cultures (1:1). Neutrophil elastase activity was measured in a fluorometer (485 nm excitation/538 nm emission). Phorbol 12-myristate 13-acetate-induced PMNs were used as positive control (PC) (AU: arbitrary unit).

[14,23]. *T. gondii* is also known to develop extracellular traps from neutrophils of several animals (mouse, cattle, sheep, cat, harbour seal, donkey, and dolphin) [16,24–28]. In this study, it was revealed that dog PMNs developed extracellular traps against *T. gondii* tachyzoites in vitro. Moreover, the DNA ornamented with MPO, NE, and histone (H3) was observed by a fluorescence microscope in dog PMNs after incubation with tachyzoites.

Microscopic examination revealed several morphological differences in the extracellular trap structures generated by PMNs. The trap structures were divided into three groups (diffuse, spread, and aggregated) based on these differences. In the form called "diffuse" extracellular trap structure (*diff*NETs), a compact spherical network with a diameter of 25–28 nm is microscopically observed. This form consists of decondensed protein-carrying antimicrobial proteins. Another form is the "spread" (*spr*NETs); it forms decondensed, protein-structured, long, thin, web-like strands of 15–17 nm in length carrying antimicrobial proteins. The "aggregated" form (*agg*NETs) is large aggregated reticulated clusters, seen as large clusters with a large number of PMNs involved. This large structure consists of extracellular chromatin-carrying granular proteins with a diameter of above 50 µm [20]. Different trap structures were reported in canine PMN–*D. immitis* microfiler and third-stage larvae cocultures [23]. In the present study, after 1-h incubation, extracellular trap structures in diffuse and spread forms (*diff*NETs and *spr*NETs) were observed in PMN-tachyzoite culture. It was also observed that a single tachyzoite was attached to the extracellular trap structures in *spr*NETs, while more than one tachyzoite was observed in *diff*NETs. The prolongation of the incubation time can have an effect on the development of NETosis structure since the amount of extracellular DNA was increased in parallel with the incubation time.

The incubation time had a different effect on the development of extracellular traps in parasite-PMN cultures. The amount of extracellular DNA released in goat and bovine neutrophils incubated with *Eimeria bovis*, *E. arloingi*, and *Cryptosporidium parvum* sporozoites increased in parallel with the incubation time [9,11,27]. The opposite has been observed in bovine PMN-*Besnoitia besnoiti* tachyzoites cultures, and the amount of extracellular DNA released during incubation did not increase in parallel with the incubation period [20]. The amount of extracellular DNA formed in the studies on *T. gondii* changed over time [24–28]. Time-dependent

increase in extracellular DNA levels against *T. gondii* tachyzoites has been reported from PMNs of mouse, sheep, cattle, cat, donkey, and dolphin [24–28]. In the present study, in accordance with the results of previous studies [24–28], the amount of extracellular DNA increased with the concentration of the parasite. However, the differences were not statistically significant ($P > 0.05$). The amount of extracellular DNA released from PMA-induced dog neutrophils (the positive group) was higher than that of the PMN-tachyzoite culture.

There have been some reports of lethal effects of extracellular traps against *T. gondii* in some animals [16,24]. However, it is not well known which chemical has lethal effects on tachyzoites during NETosis [24]. Neutrophil granules contain many antimicrobial enzymes and proteins that act on pathogens [31,32]. MPO, one of the important enzymes in the primary granules, exhibits an antimicrobial effect on pathogens both inside of the neutrophil and in the extracellular area [33]. MPO activity is reported from bovine and sheep PMNs after incubation with *T. gondii* tachyzoites [16]. The MPO level in bovine PMN is higher than that of sheep PMN during incubation and it increases up to the 100th min of incubation in bovine PMNs [16]. The MPO level increases up to the 80th min of incubation in the PMNs of donkey after incubation with *T. gondii* tachyzoites [26]. In the present study, the MPO activity tended to increase in the dog PMNs during the first 40 min of incubation. Then, the activity remained constant for 70 min and tended to decrease slowly.

Neutrophil elastase plays a role in oxygen-independent antimicrobial mechanisms [34]. The NE activity is reported from donkey PMNs after incubation with *T. gondii* tachyzoites [26]. In the present study, the NE level increased in the dog PMN-tachyzoites culture during the incubation period (150 min).

In conclusion, the development of extracellular traps against *T. gondii* in dog PMNs is reported for the first time. Time-dependent and dose-dependent relationships were observed but they were not statistically important. The MPO and NE activities were detected during the incubation of tachyzoites-PMN cocultures. It could not be determined whether the extracellular traps released from dog PMNs only mechanically immobilize or have some lethal effect on tachyzoites. This needs to be clarified in future studies. Furthermore, the differences in granular contents of the neutrophils of the dog should be demonstrated.

Acknowledgments

This study was financially supported by the Scientific Research Unit of Kirikkale University (grant no: 2017/80) as a part of Gunes Karakurt's Ph.D. thesis. We would like to thank Dr. Cahit Babur for the *T. gondii* tachyzoites.

References

1. Dubey JP. Toxoplasmosis of Animals and Humans. 2 nd ed. Boca Raton, FL: CRC Press; 2010.
2. Dubey JP, Lappin MR. Toxoplasmosis and neosporosis. In: Greene CE (editor). Infectious Diseases of the Dog and Cat, 3rd ed. St. Louis, MO, USA: Saunders Elsevier; 2006. pp: 754-775.
3. Dubey JP. Toxoplasmosis in dogs. Canine Practice 1985; 12: 7-28.
4. Ahmed BA, Goafar SM, Werich WE, Konitz CL. Relationship of *Toxoplasma* infections to other diseases in dogs. Veterinary Parasitology 1983; 12: 199-203.
5. Da Silva AV, Pezerico SB, De Lima VY, D'arc Moretti L et al. Genotyping of *Toxoplasma gondii* strains isolated from dogs with neurological signs. Veterinary Parasitology 2005; 127: 23-27.
6. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y et al. Neutrophil extracellular traps kill bacteria. Science 2004; 303: 1532-1535.
7. Branzk N, Papayannoupoulos V. Molecular mechanisms regulating NETosis in infection and disease. Seminars in Immunopathology 2013; 35: 513-530.
8. Munoz-Caro T, Hermosilla C, Silva LMR, Cortes H, Taubert A. Neutrophil extracellular traps as innate immune reaction against the emerging apicomplexan parasite *Besnoitia besnoiti*. Plos one 2014; 9: 1-9. doi: 10.1371/journal.pone.0091415
9. Silva LMR, Munoz-Caro T, Gerstberger R, Vila-Vicosa MJM, Cortes HCE et al. The apicomplexan parasite *Eimeria arloingi* induces caprine neutrophil extracellular traps. Parasitology Research 2014; 113: 2797-2807.
10. Guimares-Costa AB, Nascimento MT, Froment GS, Soares RP, Morgado FN et al. *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. Proceedings of the National Academy of Sciences of the United States of America 2009; 106: 6748-6753.
11. Behrendt JH, Ruiz A, Zahner H, Taubert A, Hermosilla C. Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite *Eimeria bovis*. Veterinary Immunology and Immunpathology 2010; 133: 1-8.
12. Gabriel C, McMaster WR, Girard D, Descoteaux A. *Leishmania donovani* promastigotes evade the antimicrobial activity of neutrophil extracellular traps. Journal of Immunology 2010; 185: 4319-4327.
13. Avila EE, Salazia N, Pulido J, Rodriguez MC, Diaz-Godinez C et al. *Entamoeba histolytica* trophozoites and lipopeptidophosphoglycan trigger human neutrophil extracellular trap. Plos One 2016; 11: 1-18. doi: 10.1371/journal.pone.0158979
14. Wei Z, Hermosilla C, Taubert A, He X, Wang X et al. Canine neutrophil extracellular traps release induced by the apicomplexan parasite *Neospora caninum* in vitro. Frontiers in Immunology 2016; 7: 1-7. doi: 10.3389/fimmu.2016.00436

15. Villagra-Blanco R, Silva LMR, Munoz-Caro T, Yang Z, Li J, Gartner U et al. Bovine polymorphonuclear neutrophils cast neutrophil extracellular traps against the abortive parasite *Neospora caninum*. Frontiers in Immunology 2017; 8: 1-11. doi: 10.3389/fimmu.2017.00606

16. Yildiz K, Gokpinar S, Gazyagci AN, Babur C, Sursal N et al. Role NETs explain the difference in host susceptibility to *Toxoplasma gondii* between sheep and cattle. Veterinary Immunology and Immunpathology 2017; 189: 1-10.

17. Fei L, Zhengkai W, Lili C, Yuhang G, Zhengtao Y et al. *Trichomonas vaginalis* triggers the release of THP-1 extracellular traps. Parasitology Research 2018; 118: 267-274.

18. Bonne-Annee S, Kerepesi LA, Hess JA, Wesolowski J, Paumet F et al. Extracellular traps are associated with human and mouse neutrophil and macrophage mediated killing of larval *Strongyloides stercoralis*. Microbes and Infection 2014; 16: 502-511.

19. Chuah C, Jones MK, Burke ML, McManus DP, Owen HC et al. Defining a pro-inflammatory neutrophil phenotype in response to *Schistosom*e eggs. Cellular Microbiology 2014; 16: 1666-1177.

20. Munoz-Caro T, Rubio RMC, Silva LM, Magdowski G, Gartner U et al. Leucocyte-derived extracellular trap formation significantly contributes to *Haemonchus contortus* larval entrapment. Parasites and Vectors 2015; 8: 1-12. doi: 10.1186/s13071-015-1219-1

21. Mendez J, Sun D, Tuo W, Xiao Z. Bovine neutrophils form extracellular traps in response to the gastrointestinal parasite *Ostertagia ostertagi*. Scientific Reports 2018; 8: 1-12. doi: 10.1038/s41598-018-36070-3

22. Lange MK, Penagos-Tabares F, Munoz-Caro T, Gartner U, Mejer H et al. Gastropod-derived haemocyte extracellular traps entrap metastrongyloid larval stages of *Angiostrongylus vasorum, Aelurostrongylus abstrusus* and *Troglostrongylus brevior*. Parasites and Vectors 2017; 10: 1-12. doi: 10.1186/s13071-016-1961-z

23. Munoz-Caro T, Conejeros I, Zhou E, Pikhovych A, Gartner U et al. *Dirofilaria immitis* microfilariae and third-stage larvae induce canine NETosis resulting in different types of neutrophil extracellular traps. Frontiers in Immunology 2018; 9 : 1-12.

24. Abi Abdallah DS, Lin C, Ball CJ, King MR, Duhamel GE et al. *Toxoplasma gondii* triggers release of human and mouse neutrophil extracellular traps. Infection and Immunity 2011; 80: 768-777.

25. Sursal N, Cakmak A, Yildiz K. Kedi polimorf nükleer lökositlerin in vitro ortamda *Toxoplasma gondii*'ye karşı geliştirdiği hücre dışı tuzak oluşumunun araştırılması. PhD, Ankara Üniversitesi Sağlık Bilimleri Enstitüsü, Ankara, Turkey, 2017 (in Turkish with an abstract in English).

26. Yildiz K, Gokpinar S, Sursal N, Babur C, Ozen D et al. Extracellular trap formation by donkey polymorphonuclear neutrophils against *Toxoplasma gondii*. Journal of Equine Veterinary Science 2019; 73: 1-9.

27. Reichel M, Munoz-Caro T, Contreras GS, Garcia AR, Magdowski G et al. Harbour seal (*Phoca vitulina*) PMN and monocytes release extracellular traps to capture the apicomplexan parasite *Toxoplasma gondii*. Developmental and Comparative Immunology 2015; 50: 106-115.

28. Imlau M, Conajeros I, Munoz-Caro T, Zhoua E, Gartner U et al. Dolphin-derived NETosis results in rapid *Toxoplasma gondii* tachyzoite ensnarement and different phenotypes of NETs. Developmental and Comparative Immunology 2020; 103: 1-9. doi: 10.1016/j.dci.2019.103527

29. Sursal N, Cakmak A, Yildiz K. Neutrophil isolation from feline blood using discontinuous Percoll dilutions. Tierarztliche Praxis Ausgabe K Kleintiere Heimtiere 2018; 46: 399-402.

30. Jeffery U, Kimura K, Gray R, Lueth P, Bellaire B et al. Dogs cast NETs too: Canine neutrophil extracellular traps in health and immune-mediated hemolytic anemia. Veterinary Immunology and Immunopathology 2015; 168: 262-268.

31. Guimares-Costa AB, Nascimento MT, Wardini AB, Pinto DA, Silva LH et al. ETosis: A microbicidal mechanism beyond cell death. Journal of Parasitology Research 2012; 2012: 1-11. doi: 10.1155/2012/929743.

32. Mesa MA, Vasquez G. NETosis. Autoimmun Disorders 2013; 1: 1-7.

33. Harris JR. Blood Cell Biochemistry. Volume 3: Lymphocytes and Granulocytes. Newyork, USA: Springer Science+Business Media; 1993.

34. Weiss DJ, Wardrop KJ. Schalm's Veterinary Hematology. 6th ed. Iowa, USA: Blackwell Publishing; 2011.