Title:

Sex difference in plasma deoxyribonuclease activity in rats

Short title:

DNase activity sex difference

Authors:

Ľubica Janovičová[1], Barbora Gromová[1], Diana Drobná[1], Barbora Konečná[1], Emese Renczés[1], Veronika Borbélyová[1], Július Hodosy[1, 2], Peter Celec[1, 3, 4*]

Affiliations:

[1] Institute of Molecular Biomedicine, Faculty of Medicine, Comenius University, Bratislava, Slovakia

[2] Institute of Physiology, Faculty of Medicine, Comenius University, Bratislava, Slovakia

[3] Institute of Pathophysiology, Faculty of Medicine, Comenius University, Bratislava, Slovakia

[4] Department of Molecular Biology, Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia

* Corresponding author

Assoc. Prof. Peter Celec, MD, Dipl. Ing., MSc, PhD, MPH

Institute of Molecular Biomedicine

Faculty of Medicine

Comenius University

Bratislava

Slovakia

E-mail: petercelec@gmail.com

Summary

Extracellular DNA (ecDNA) activates immune cells and is involved in the pathogenesis of diseases associated with inflammation such as sepsis, rheumatoid arthritis or metabolic syndrome. DNA can be cleaved by deoxyribonucleases (DNases), some of which are secreted out of cells. The aim of this experiment was to describe plasma DNase activity in relation to extracellular DNA in adult rats, to analyse potential sex differences and to prove whether they are related to endogenous testosterone. Adult Lewis rats (n=28) of both sexes were included in the experiment. Male rats were gonadectomized or sham-operated and compared to intact female rats. Plasma ecDNA and DNase activity were measured using fluorometry and single radial enzyme diffusion assay, respectively. Concentrations of nuclear ecDNA and mitochondrial ecDNA were determined using real-time PCR. Females had 60% higher plasma DNase activity than males (p=0.03). Gonadectomy did not affect plasma DNase in males. Neither the concentration of total ecDNA, nor nuclear or mitochondrial DNA in plasma differed between the groups. No significant correlations between DNase and ecDNA were found. From previous studies on mice, it was expected, that male rats will have higher DNase activity. In contrast, our study in rats showed the opposite sex difference. This sex difference seems not to be caused by endogenous testosterone. Interestingly, no sex differences were observed in plasma ecDNA suggesting a complex or missing association between plasma ecDNA and DNase. The observed sex difference in plasma DNase should be taken into account in animal models of ecDNA-associated diseases.

Key words: cell-free DNA, nuclease activity, NETosis, experimental animals

Introduction

Extracellular DNA (ecDNA) is a term covering all DNA molecules outside of cells. Intracellular nucleic acids can be released from cells during apoptosis or necrosis [1]. A characteristic feature of ecDNA is the specific pattern of fragmentation. The average fragment length is approximately 166 bp. It is thought to represent the length of a single turn of DNA wrapped around histones [2]. EcDNA can originate from nucleus or mitochondria and both can cause an activation of immune cells via Toll-like receptor 9 signalisation, but also other DNA sensors [3].

Deoxyribonucleases (DNases) are enzymes which can cleave DNA. Activity of these enzymes depends on their expression and, thus, quantity, but also on the presence of activators and inhibitors such as divalent ions of calcium and magnesium or actin, respectively. DNase I is secreted outside of cells, where it can cleave ecDNA. In animal models of diseases such as ischemic-reperfusion injury, liver failure, sepsis and colitis, exogenous DNase I helps to prevent tissue damage and improves survival [4-7]. The positive effects of DNase I administration are likely mediated by the cleavage of the DNA mesh in neutrophil extracellular traps preventing further inflammation and coagulation [8]. Sex differences in disease severity were described in a model of liver failure with male rats having a worse prognosis [9]. Understanding these differences and the underlying mechanisms potentially involving DNase is crucial when selecting a disease model, model organism and translating the results to humans.

Sex differences were identified in DNase activity in some tissues in mice but not yet in rats [10]. There are several possible explanations why there are sex differences in DNase activity. Sex hormones may be responsible for the regulation of DNase production or activity. Body composition might be responsible for sex differences in ecDNA or DNase activity. The higher body fat has a partial association with higher ecDNA concentration [11]. The reason might be the sex difference in ecDNA removal. Sex differences were observed in brain damage and subsequent behavioral impairments in both, mice, and rats [12,13]. The causes and consequences of these sex differences are unknown. As ecDNA is involved in the pathogenesis of autoimmune diseases, sepsis and other pathologies, sex differences in ecDNA or DNase could be of importance for the understanding of sex differences in disease risk in humans or variability in disease models in experimental animals. Therefore, our aim was to describe plasma DNase activity and ecDNA in plasma of adult male and female rats. In addition, using castration we wanted to prove whether the expected sex differences are due to the action of androgens.

Methods

Animals and procedures

The experiment was approved by the local Ethics Committee of the Institute of Molecular Biomedicine. All experiments were performed in accordance with the national legislation. All surgery was performed under ketamine: xylazine anaesthesia cocktail and all efforts were made to minimize suffering. Twenty-eight healthy adult Lewis rats (Anlab, Prague, Czech Republic) of both sexes were included in experimental procedures and were housed under standard condition with stable temperature, 12/12-hour light/dark cycle and *ad libitum* access to standard pellet diet (KMK20, EYPY, Czech republic) and water. The animals were divided into 3 groups: control males which were sham-operated, gonadectomized males and control females. Rats were gonadectomized or sham operated on postnatal day 29-31. At the age of 18 months blood was collected into both, EDTA- and heparin-containing tubes from the aorta in ketamine: xylazine anaesthesia. Blood was centrifuged at 2000g for 5 min. EDTA plasma was centrifuged again to remove cell debris at 16000g for 10 min at 4^{o}C. The supernatant of plasma samples was stored at -20^{o}C until analysis.

Testosterone measurement

Testosterone concentration in plasma was measured using a commercially available ELISA kit (DRG Diagnostic, Marburg, Germany) to confirm the effect of castration. Measurement was carried out according to the standard protocol provided by the manufacturer.

DNase activity measurement

DNase activity was measured in heparin plasma using the modified single radial enzyme diffusion assay. Briefly, agarose gels were prepared with final concentration of solutes: 1 mM $CaCl_2$, 1 mM $MgCl_2$ and DNA isolated from rat liver (0.5 mg/ml). Ethidium bromide was replaced with the fluorescent dye Goodview (SBS Genetech, Beijing, China). DNase activity was recalculated based on serial dilutions of RNase-free DNase I (Qiagen, Hilden, Germany). After 18 hours of incubation, gels were scanned using iBOX (Vision works LP Analysis Software, UVP, Upland, CA, USA). Diameters of cleared circles on the gel were measured using ImageJ software.

Quantification of extracellular DNA

EDTA plasma was used for the isolation of ecDNA using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). Concentration of ecDNA was measured using a fluorometric method with the Qubit 3.0 fluorometer and Qubit dsDNA HS kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantification of DNA using PCR was conducted for both, nuclear and mitochondrial DNA. PCR was performed on the Mastercycler realplex 4 (Eppendorf, Hamburg, Germany). The reaction was carried out with 7.5 µl of SYBR Green master mix (Qiagen, Hilden, Germany), 0.3 µl of forward primer (10 µM), 0.3 µl of reverse primer (10 µM), 3.9 µl of millipore water and 3 µl of template. For nuclear DNA (ncDNA) forward 5'- GAAATCCCCTGGAGCTCTGT -3' and reverse 5'- CTGGCACCAGATGAAATGTG -3' primers (GADPH) [14], for mitochondrial DNA (mtDNA) forward- 5'-CCT CCC ATT CAT TAT CGC CGC CCT TGC-3' and reverse 5'-GTC TGG GTC TCC TAG TAG GTC TGG GAA-3' primers (211 bp mitochondria fragment) [15] (Eurofins Genomics, Ebersberg, Germany) were used. The PCR program consisted of initial DNA denaturation at 95^{o}C for 15 min, 40 cycles of denaturation at 94^{o}C for 15 sec, annealing at 60^{o}C for 30 sec and elongation at 72^{o}C for 30 sec followed by melting curve analysis to confirm specificity of the PCR. Analysis of distribution of ecDNA fragments length of selected samples from males and castrated males was done using capillary electrophoresis and the BIABooster technology (Adelis, Grabels, France) [16].

Statistics

Data were analysed using GraphPad Prism 6 (La Jolla, CA, USA). Comparison of groups was carried out using ANOVA with Bonferroni corrected t-test. Correlation analysis was conducted with the Pearson correlation test. P-values less than 5% were considered significant. Results are presented as mean + standard deviation.

Results

Plasma testosterone was 0.85+0.24 nmol/l in females and in 4.71+2.95 nmol/l males ($t=6.76$, $p<0.001$) confirming the physiological sex difference. Males after castration had testosterone concentrations of 0.68+0.18 nmol/l which is significantly lower in comparison to control males ($t=6.72$, $p<0.001$) and similar to females.

A sex difference in DNase activity was found in plasma. Female rats had on average 60% higher average DNase activity compared to control male rats. There was no difference in DNase activity between control males and castrated males ($F=3.87$, $p=0.03$; females vs males $t=2.66$ $p=0.03$, males vs castrated males $t=0.31$ $p>0.99$) (Figure 1A).

Concentration of total plasma ecDNA was measured in castrated males, control males and control females. No differences between groups were observed in concentrations of total ecDNA ($F=0.57$, $p=0.57$) (Figure 1B). Quantification of ecDNA by PCR also showed no sex difference. Groups did not differ in plasma ncDNA ($F=0.12$, $p=0.89$) or mtDNA ($F=1.90$, $p=0.17$) (Figure 1C+D). High interindividual variability was observed in both, ncDNA (CV: 107%) and mtDNA (CV: 103%). Fragmentation analysis of ecDNA showed no difference in ecDNA size profile between males and castrated males. In both groups the highest peak was around 100-200 bp length with similar distribution of DNA fragments between the groups (Interaction: $F=0.02$, $p>0.99$; Fragment length: $F=15.01$, $p<0.001$; Groups: $F=0.06$; $p=0.81$, Figure 1E).

No significant correlation of DNase activity with total ecDNA in plasma was found ($r=-0.56$, $p=0.09$, Figure 2A). Similarly, no correlations of DNase activity and ncDNA ($r=-0.61$, $p=0.08$, Figure 2B) or mtDNA were found ($r=-0.36$, $p=0.31$, Figure 2C). In male rats, concentration of ecDNA and DNase activity did not correlate ($r=0.10$, $p=0.75$, Figure 2D). No association was found for DNase activity and mtDNA ($r=0.11$, $p=0.72$, Figure 2E) or ncDNA in male rats ($r=-0.37$, $p=0.24$, Figure 2F). DNase activity did not correlate with ecDNA in castrated male rats ($r=0.18$, $p=0.64$, Figure 2G). A correlation was not observed between DNase activity and neither ncDNA ($r=0.13$, $p=0.74$, Figure 2H) nor mtDNA in castrated males ($r=0.55$, $p=0.13$, Figure 2I).

Discussion

In our study, we have found a sex difference in plasma DNase activity in rats. Surprisingly, female rats had higher DNase activity compared to male rats. The opposite sex difference was expected based on the study where higher DNase activity was observed in liver, kidney and urine from male mice [10]. It is clear that there are major interspecies differences in DNase activity in plasma [17]. The origin of plasma DNase activity should be understood before further animal experiments testing the effects of exogenous or endogenous DNase on models of human diseases are conducted.

No differences between groups were observed in ecDNA of any subcellular origin. However, the concentrations of ecDNA, ncDNA and mtDNA showed high interindividual variability. Interestingly, no association was found between plasma DNase activity and ecDNA quantified using two different methods – spectrofluorometry and PCR. This could be explained by the fact that total DNA measured spectrofluometrically includes short fragments that are not identified using real time PCR. It is unclear whether these short fragments are of biological importance. It is expected that concentration of ecDNA in plasma can be to some degree affected by DNases. Our results show that there is no simple linear negative association between ecDNA and DNase. This could be explained by the likely protection of plasma ecDNA by nucleosomes, which might be of importance especially for ncDNA [18]. On contrary, mtDNA does not interact with histones, but in our study mtDNA does not correlate with DNase activity either. One of recent reports suggests that plasma ecDNA could be hidden in extracellular vesicles such as exosomes, which might protect both, ncDNA and mtDNA from degradation by plasma DNase activity [19]. Whether this is true for plasma DNA of experimental animals is currently not clear.

Research of ecDNA is focusing mostly on diagnostic applications, but relatively little is known about its biology, for example the sources of variability of its concentrations in plasma. On the other hand, the quantity of plasma ecDNA is being tested as a potential disease marker e.g. in sepsis [20]. Free ecDNA outside of exosomes and free from histones and other DNA-binding proteins could be cleaved by plasma DNase. However, a single study describing the relationship between concentration of ecDNA and DNase activity showed that DNase I knock-out mice do not have altered fragmentation of ecDNA in plasma [21]. Our results confirm this finding and show that there is no linear association between ecDNA and DNase activity in mouse plasma. Recently, two DNases were shown to be essential for survival of mice in induced neutrophilia that increases ecDNA. DNase I and DNase IL3 cooperate to manage ecDNA concentrations in the extracellular space [22]. Our analysis covers the ability of plasma to cleave DNA rather than focusing on specific DNases. Other clearance mechanisms should also be

considered, for example macrophages, which digest debris after cell death were shown to aid ecDNA removal [23]. It is possible that healthy animals have very little completely free ecDNA in plasma since no major pathological mechanisms are activated that could increase its production. This speculation requires many additional experiments especially with experimental animals suffering from a disease model such as sepsis or trauma.

Sex differences in plasma DNase activity could be caused by sex hormones modulating DNase activity. However, we have found no differences between control and castrated males, which suggests that testosterone is not the factor affecting plasma DNase activity in adult rats. So, an alternative to testosterone in males could be the effect of estrogens in females that will be tested in our future experiments. Male rat castration leads to low grade inflammation that is not affected by replacement therapy [24]. A similar experiment on females with ovariectomy showed similar results [25]. This inflammation could explain an increase in ecDNA, but neither plasma ecDNA nor DNase activity were affected by castration in our experiment. Besides sex hormones, genetic factors could underlie sex differences in plasma DNase activity. Despite inactivation of one X chromosome in females, gene dosage could affect DNase activity. DNase X is located on the X chromosome. However, this DNase is highly expressed in skeletal muscles and it is not known to affect the plasma DNase activity [26]. DNases were shown to be regulated by EndoG. This enzyme is secreted from mitochondria and can cleave both, DNA and RNA [27]. Whether EndoG regulation or its expression is sex-specific is currently unknown.

In mice and humans, males have higher DNase activity in plasma. This known sex difference is driving the interest in the research of sex differences in relation to sex hormones. Studies previously described DNase activity in many organs among which were testes, ovaries and prostate. Gonadectomy in male mice induces apoptosis in cells of prostate and increase in DNA-fragmentation [28]. The gonadectomy is linked to muscle atrophy which is caused by autophagy. A study showed that fasting in castrated rats induces muscle atrophy linked to mitochondrial stress [29]. The muscle atrophy could be a source of DNase I inhibitor, actin [30]. Even if DNase activity was determined by sex hormones, it still could be inhibited in castrated rats, therefore, not changing the DNase activity in their plasma. The gonadectomy was described to lead to accumulation of DNase I in prostate epithelial cells. It is the DNase I or its isoforms that act to cleave ecDNA in cells preparing to undergo apoptosis [31].

One of the limitations of our analysis is that the DNase 1L3 is inhibited by heparin in collection tubes. The choice of anticoagulant may affect the DNase activity. The blood for DNase activity measurement cannot be

collected in EDTA-containing tubes as EDTA is an inhibitor of DNase I. Another option is to collect serum which is unsuitable for analysis of ecDNA because during the coagulation process ecDNA is released. If ecDNA is released so can be other co-factors or proteins altering DNase activity in plasma.

In conclusion, according to our knowledge this is the first study showing a sex difference in plasma DNase activity in rats. On contrary, plasma ecDNA of both, nuclear and mitochondrial origin, is comparable in both sexes. What are the causes for the observed sex difference remains to be studied, because castration did not affect plasma DNase activity. An opposite sex difference has been described in mice and large interspecies differences in DNase activity exist [32]. Nevertheless, from the experimental rodents, rats in contrast to mice have a DNase activity that is similar to humans. Thus, it is likely that rats rather than mice should be used to model ecDNA-associated human diseases. It is crucial to uncover the determinants of plasma DNase activity and the mechanism of the observed sex difference. This might shed light on the pathogenesis of sepsis, trauma complications, but also other pathologies. Additional studies are required to describe the regulation of plasma DNase activity and/or ecDNA concentrations. Their results might be helpful in the understanding of the pathogenesis of ecDNA-associated diseases.

Acknowledgements

The research was supported by the Slovak Research and Development Agency (APVV-16-0273).

Conflict of interest

None to declare.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

References

1. Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. *Clinica chimica acta; international journal of clinical chemistry.* 2001;313(1-2):139-142.
2. Chandrananda D, Thorne NP, Bahlo M. High-resolution characterization of sequence signatures due to non-random cleavage of cell-free DNA. *BMC medical genomics.* 2015;8:29.
3. Speranskii AI, Kostyuk SV, Kalashnikova EA, Veiko NN. [Enrichment of extracellular DNA from the cultivation medium of human peripheral blood mononuclears with genomic CpG rich fragments results in increased cell production of IL-6 and TNF-a via activation of the NF-kB signaling pathway]. *Biomeditsinskaia khimiia.* 2016;62(3):331-340.
4. Albadawi H, Oklu R, Raacke Malley RE, et al. Effect of DNase I treatment and neutrophil depletion on acute limb ischemia-reperfusion injury in mice. *Journal of vascular surgery.* 2016;64(2):484-493.
5. Babickova J, Conka J, Janovicova L, Boris M, Konecna B, Gardlik R. Extracellular DNA as a Prognostic and Therapeutic Target in Mouse Colitis under DNase I Treatment. *Folia biologica.* 2018;64(1):10-15.
6. Mai SH, Khan M, Dwivedi DJ, et al. Delayed but not Early Treatment with DNase Reduces Organ Damage and Improves Outcome in a Murine Model of Sepsis. *Shock.* 2015;44(2):166-172.
7. Vokalova L, Laukova L, Conka J, et al. Deoxyribonuclease partially ameliorates thioacetamide-induced hepatorenal injury. *American journal of physiology Gastrointestinal and liver physiology.* 2017;312(5):G457-G463.
8. Delgado-Rizo V, Martinez-Guzman MA, Iniguez-Gutierrez L, Garcia-Orozco A, Alvarado-Navarro A, Fafutis-Morris M. Neutrophil Extracellular Traps and Its Implications in Inflammation: An Overview. *Frontiers in immunology.* 2017;8:81.
9. Koblihova E, Mrazova I, Vanourkova Z, Maxova H, Ryska M, Fronek J. Sex-linked differences in the course of thioacetamide-induced acute liver failure in Lewis rats. *Physiol Res.* 2020;69(5):835-845.
10. Koizumi T. Tissue distribution of deoxyribonuclease I (DNase I) activity level in mice and its sexual dimorphism. *Experimental animals.* 1995;44(3):181-185.
11. Camuzi Zovico PV, Gasparini Neto VH, Venancio FA, et al. Cell-free DNA as an obesity biomarker. *Physiol Res.* 2020;69(3):515-520.
12. Riljak V, Lastuvka Z, Myslivecek J, Borbelyova V, Otahal J. Early postnatal hypoxia induces behavioral deficits but not morphological damage in the hippocampus in adolescent rats. *Physiol Res.* 2020;69(1):165-179.
13. Lastuvka Z, Borbelyova V, Janisova K, Otahal J, Myslivecek J, Riljak V. Neonatal hypoxic-ischemic brain injury leads to sex-specific deficits in rearing and climbing in adult mice. *Physiol Res.* 2020;69(Suppl 3):S499-S512.
14. Hu Q, Wood CR, Cimen S, Venkatachalam AB, Alwayn IP. Mitochondrial Damage-Associated Molecular Patterns (MTDs) Are Released during Hepatic Ischemia Reperfusion and Induce Inflammatory Responses. *PloS one.* 2015;10(10):e0140105.
15. Rooney JP, Ryde IT, Sanders LH, et al. PCR based determination of mitochondrial DNA copy number in multiple species. *Methods Mol Biol.* 2015;1241:23-38.
16. Andriamanampisoa CL, Bancaud A, Boutonnet-Rodat A, et al. BIABooster: Online DNA Concentration and Size Profiling with a Limit of Detection of 10 fg/muL and Application to High-Sensitivity Characterization of Circulating Cell-Free DNA. *Anal Chem.* 2018;90(6):3766-3774.
17. Konecna B, Laukova L, Vlkova B. Immune activation by nucleic acids: A role in pregnancy complications. *Scandinavian journal of immunology.* 2018;87(4):e12651.

18. Ljungman M, Hanawalt PC. Efficient protection against oxidative DNA damage in chromatin. *Molecular carcinogenesis.* 1992;5(4):264-269.
19. Fernando MR, Jiang C, Krzyzanowski GD, Ryan WL. New evidence that a large proportion of human blood plasma cell-free DNA is localized in exosomes. *PloS one.* 2017;12(8):e0183915.
20. Clementi A, Virzi GM, Brocca A, et al. The Role of Cell-Free Plasma DNA in Critically Ill Patients with Sepsis. *Blood purification.* 2016;41(1-3):34-40.
21. Cheng THT, Lui KO, Peng XL, et al. DNase1 Does Not Appear to Play a Major Role in the Fragmentation of Plasma DNA in a Knockout Mouse Model. *Clinical chemistry.* 2018;64(2):406-408.
22. Jimenez-Alcazar M, Rangaswamy C, Panda R, et al. Host DNases prevent vascular occlusion by neutrophil extracellular traps. *Science.* 2017;358(6367):1202-1206.
23. Nakazawa D, Shida H, Kusunoki Y, et al. The responses of macrophages in interaction with neutrophils that undergo NETosis. *Journal of autoimmunity.* 2016;67:19-28.
24. Chin KY, Ima-Nirwana S. The Effects of Testosterone Deficiency and Its Replacement on Inflammatory Markers in Rats: A Pilot Study. *International journal of endocrinology and metabolism.* 2017;15(1):e43053.
25. Benedusi V, Martini E, Kallikourdis M, Villa A, Meda C, Maggi A. Ovariectomy shortens the life span of female mice. *Oncotarget.* 2015;6(13):10801-10811.
26. Shiokawa D, Matsushita T, Shika Y, Shimizu M, Maeda M, Tanuma S. DNase X is a glycosylphosphatidylinositol-anchored membrane enzyme that provides a barrier to endocytosis-mediated transfer of a foreign gene. *The Journal of biological chemistry.* 2007;282(23):17132-17140.
27. Zhdanov DD, Fahmi T, Wang X, et al. Regulation of Apoptotic Endonucleases by EndoG. *DNA and cell biology.* 2015;34(5):316-326.
28. Kyprianou N, English HF, Isaacs JT. Activation of a Ca2+-Mg2+-dependent endonuclease as an early event in castration-induced prostatic cell death. *Prostate.* 1988;13(2):103-117.
29. Rossetti ML, Steiner JL, Gordon BS. Increased mitochondrial turnover in the skeletal muscle of fasted, castrated mice is related to the magnitude of autophagy activation and muscle atrophy. *Mol Cell Endocrinol.* 2018;473:178-185.
30. Lazarides E, Lindberg U. Actin is the naturally occurring inhibitor of deoxyribonuclease I. *Proceedings of the National Academy of Sciences of the United States of America.* 1974;71(12):4742-4746.
31. Rauch F, Polzar B, Stephan H, Zanotti S, Paddenberg R, Mannherz HG. Androgen ablation leads to an upregulation and intranuclear accumulation of deoxyribonuclease I in rat prostate epithelial cells paralleling their apoptotic elimination. *J Cell Biol.* 1997;137(4):909-923.
32. Konecna B, Sysak R, Kacerovsky M, Celec P, Vlkova B. Deoxyribonuclease activity in plasma of pregnant women and experimental animals. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet.* 2018;31(13):1807-1809.

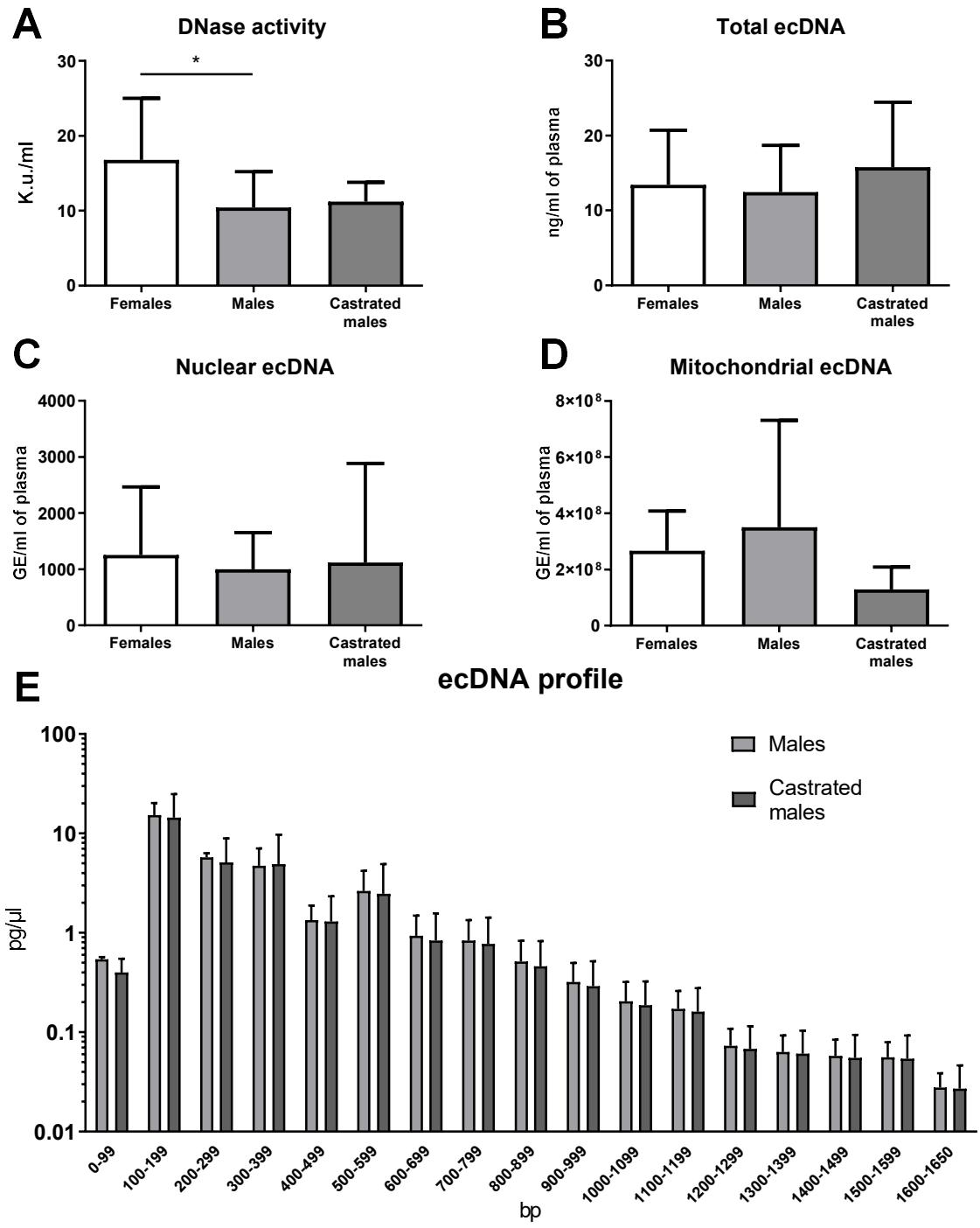
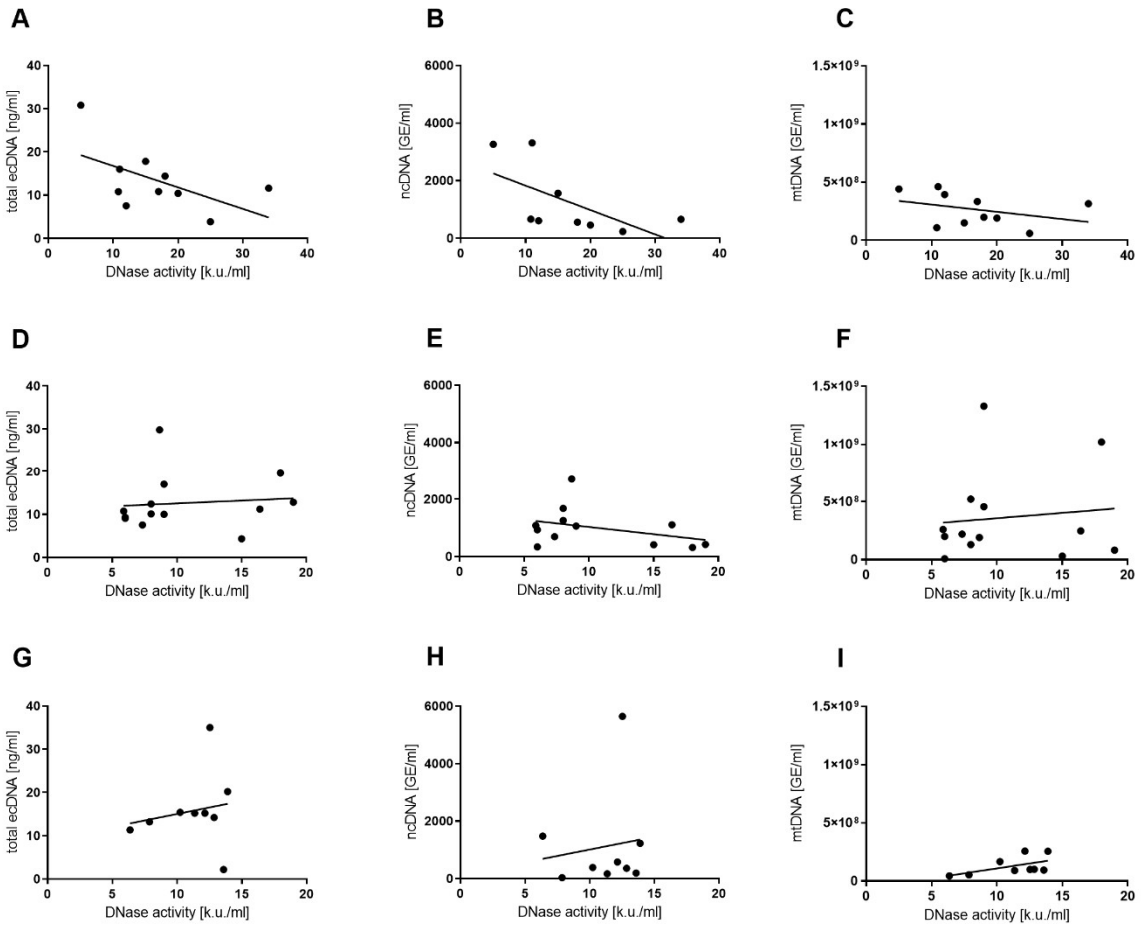


Figure 1. DNase activity and ecDNA concentration in female, male and castrated male rats. (A) Significant differences in DNase activity were found between males and females but not between control males and castrated males. (B) No differences were observed between groups in concentration of total ecDNA. Similarly, no difference was found in concentration of (C) ncDNA and (D) mtDNA between groups. (E) Analysis of

ecDNA profile of the distribution of DNA fragment lengths. No difference was observed in the length distribution of DNA fragments between males and castrated males. The results are shown as mean + standard deviation.



326

327 **Figure 2.** Correlation of DNase activity with total ecDNA, ncDNA and mtDNA. No statistically significant
328 correlations were found in any of the experimental groups. In females, no correlation of DNase with (A) ecDNA,
329 (B) ncDNA and (C) mtDNA was observed. In control males, no correlation of DNase with (D) ecDNA (F),
330 mtDNA and (E) ncDNA was observed. Similarly, in castrated males, no significant correlation of DNase activity
331 with (G) ecDNA, (H) ncDNA and (I) mtDNA was found.