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ANIMAL GENETICS

Influence of Acetylcysteine on Cytogenetic Effects of Etoposide in Mouse Oocytes

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Abstract—The influence of *N*-acetylcysteine (ACC) on the cytogenetic effects of etoposide in F_1 CBA × C57BL/6 mice was studied. Etoposide introduced intraperitoneally in doses of 10, 20, 40, and 60 mg/kg has a dose-dependent clastogenic activity and has an aneugenic effect with the induction of mainly hypohaploid oocytes. ACC significantly decreases the aneugenic and clastogenic activity of etoposide (20 mg/kg) in oocytes of 6-, 9-, and 12-week-old mice during triple introduction at a dose 200 mg/kg *per os*. The most pronounced anticlastogenic ACC activity (an 80% decrease) was registered in 9-week-old females; a 100% decrease in aneugenesis was detected in 6-week-old female mice.

Keywords: etoposide, *N*-acetylcysteine, chromosome aberrations, aneuploidy, oocytes, mice

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INTRODUCTION

The overwhelming majority of studies on the estimation of mutagenic and mutagen-modifying activity of natural and synthetic compounds are conducted in in vitro tests or in vivo tests on somatic cells [1, 2]. Studies in germ cells are rare, which is mainly caused by the limitation of existing widely used techniques. The methods that have been verified to date are complicated and laborious and are not enough reliable due to the high probability of false results [2]. This determined the need for a search for and development of new techniques of registering mutagenic and mutagen-modifying effects in germ cells. In particular, a new cytogenetic technique of registering aneuploidy and structural chromosome violations in mouse oocytes was recently suggested [3]; approbation and verification in experiments with known mutagens and antimutagens is a necessary stage of its implementation.

In the present work, *N*-acetylcysteine (ACC) was selected as an antimutagen; it is widely used in clinical practice as a mucolytic agent with cytoprotective activity associated with antioxidant properties of the preparation [4, 5]. Antimutagenic and anticarcinogenic ACC properties were demonstrated in multiple in vivo experiments on somatic cells relative to genotoxicants with different mechanisms of action [6].

Etoposide (an antitumor drug belonging to the class of topoisomerase II inhibitors that is widely used in clinics) was selected as a mutagen. Etoposide use is associated with a high risk of mutagenesis and the development of secondary tumors [7].

The ability of ACC to decrease lethal mutations induced by etoposide in male mice was demonstrated in previous studies [8]; no estimation effects in oocytes was previously conducted.

The goal of the present work was to study the influence of ACC on the cytogenetic effects of etoposide in mouse oocytes.

MATERIALS AND METHODS

The study was conducted on 6-, 9-, and 12-week-old females of F_1 CBA × C57BL/6 mice (Stolbovaya nursery). The animals were kept in a vivarium in the Zakusov Research Institute of Pharmacology according to the sanitary standards provided by the Laboratory Practice Rules (Order of the Ministry of Health and Social Development of Russian Federation from August 23, 2004, no. 708n) with free access to water and a balanced, briquetted, granular mixed feed from the MEST company (Russia).

Superovulation in the mice of all experimental groups was caused by means of an intraperitoneal injection of 5 ME of gonadotropin of pregnant mare serum (Folligon, Holland) and 5 ME of human chorionic gonadotropin (hCG) (Moscow Endocrine Plant), with a 48-h interval between injections.

Etoposide at doses of 10, 20, 40, and 60 mg/kg (Etoposide-Lens, Verofarm) was introduced to females of 6-week-old mice once, intraperitoneally, simultaneously with the hCG injection. An equivalent amount of physiological solution was introduced to mice in the negative control group simultaneously with the CG injection.

The experiments with modification of etoposide effects were conducted on 6-, 9-, and 12-week-old

Table 1. Induction of numerical chromosome violations in mouse oocyte by etoposide

Experimental conditions	Oocytes at meiosis MI stage, %	Metaphases (%) with chromosome number			Polyhaploid metaphases, %	Metaphases with PSSC, %
		$n < 20$	$n = 20$	$n > 20$		
Negative control	0.5	0	100	0	0	0
Etoposide 10 mg/kg	2.0	3.6	94.4	1.5	0.5	0
Etoposide 20 mg/kg	1.0	6.6**	91.4	2.0*	0	5.1*
Etoposide 40 mg/kg	1.5	11.7**	80.2	2.0*	1.0	24.9**
Etoposide 60 mg/kg	3.5*	n/e	n/e	n/e	2.0*	n/e

*, $p < 0.05$; **, $p < 0.01$ as compared with negative control; n/e, not estimated.

female mice. ACC at a dose 200 mg/kg was introduced orally three times, simultaneously with the injection of gonadotropin of pregnant mare serum, 24 h after and together with the etoposide injection. The selection of the ACC dose was based on literature data [8].

Each experimental group included 10–15 animals. Altogether, four series of experiments were carried out.

The obtaining of the micropreparations and the cytogenetic analysis of oocytes were conducted according to a previously developed technique [3]. Animals were killed 17 h after CG injection by dislocation of the cervical vertebrae. Oviducts were extracted and placed in a drop of EKO1 Ooklin medium (PanEko, Russia) (preliminarily heated to 37°C) in a Petri dish. The oviduct ampoule was broken under a stereomicroscope (Stemi DV4, Carl Zeiss) by means of a preparation needle. Oocytes released in the oocyte–cumulus complex were transferred to a medium containing 150 ME/mL hyaluronidase (type II, Sigma). Oocytes released from cumulus cells were selected 20 min later by EZ-Grip® micropipette (RI, England) and transferred through 6–8 drops of fresh medium for washing from the enzyme and follicular cells. Removal of the zona pellucida was carried out by oocyte treatment with 0.005% collagenase solution (Type I, Sigma) for 20 min at room temperature. Oocytes were then placed in a fresh portion of medium to be washed free of collagenase (as described above).

Hypotonic treatment was conducted in buffered 0.4% KCl solution (10 mM HEPES, 1 mM $MgCl_2$, pH 7.2) for 15 min at room temperature. At the end, 20–25 oocytes were transferred to wells in a slide with 30 μL of fixative (1% paraformaldehyde, 0.2% Triton-X, 1.5 mM dithiothreitol, pH 9.2). Three-well slides for immunofluorescence with hydrophobic teflon surface were used (Immuno-cell, Belgium). Micropreparations were placed in a moist chamber for 2 h, after which they were dried at 37°C.

Micropreparations were dyed by fluorescent Hoechst 33258 dye (0.5 μg/mL in phosphate–saline buffer) for 10 min. The microscopy was carried out on an epifluorescent AxioImager M2 microscope (Carl Zeiss, Germany) under oil immersion at a magnification of ×1000. Digital images of metaphase plates were obtained with an AxioCam MRm digital camera.

At least 200 metaphases were analyzed for each experimental group. Numerical (aneu- and polyploidy) and structural violations (chromatid and chromosome fragments, exchanges of different types), as well as the premature separation of sister chromatids (PSSC), were estimated. Metaphases with multiple aberrations(more than five per cell) were isolated into a separate group. The statistical treatment of the data was conducted with the χ^2 criterion.

RESULTS

The cytogenetic effects of etoposide were studied in the first series of experiments in mouse oocytes in order to determine the optimal mutagen dose to estimate the modifying effect of ACC.

Data from the experiments on the estimation of the aneugenic effects of etoposide are presented in Table 1.

No oocytes with numerical chromosome violations were detected in the control animals, which is in agreement with previously obtained data on the spontaneous aneuploidy level in this mouse strain [3].

After the introduction of etoposide at a dose 10 mg/kg, 3.6 and 1.5% of hypohaploid ($n < 20$) and hyperhaploid ($n > 20$) oocytes, respectively, were detected; however, differences with the control indices were statistically insignificant.

Etoposide induced an oocyte yield with a hypohaploid set of chromosomes at doses 20 and 40 mg/kg of up to 6.6 and 11.7%, respectively. The percentage of oocytes with a hyperhaploid set of chromosomes was also significantly higher; an index of 2% was registered for both doses. PSSC was observed in 5.1 and 24.9% oocytes for etoposide doses of 20 and 40 mg/kg, respectively.

A significant increase in the percentage of oocytes with meiotic arrest (up to 3.5%), as well as oocytes with a polyploidy set of chromosomes ($2n$, $3n$), was detected after the introduction of etoposide at a dose of 60 mg/kg. The numerical analysis of the chromosomes and PSSC was complicated due to the high level of chromosomal structural violations (Table 2).

Table 2. Induction of structural chromosome aberrations in mouse oocytes by etoposide

Experimental conditions	Per 100 cells				Total damaged metaphases ($M \pm m$), %
	chromatid fragments	chromosome fragments	exchanges	cells with MD	
Negative control	0	0	0	0	0
Etoposide 10 mg/kg	5.4	0	0	0	4.7* ± 1.5
Etoposide 20 mg/kg	25.3	0	3.0	0	19.2** ± 2.8
Etoposide 40 mg/kg	89.3	0.5	5.6	9.6	59.4** ± 3.5
Etoposide 60 mg/kg	76.7	0.5	7.3	47.2	82.4** ± 2.7

MD, multiple (>5) damages of chromosomes (for Tables 2, 4).
*, $p < 0.05$; **, $p < 0.001$ as compared with negative control.

The data on the analysis of chromosome aberrations in oocytes are presented in Table 2. No oocytes with chromosome structural violations were detected in animals from the negative control group. Etoposide induced chromosome aberrations in oocytes depending on the dose (up to 4.7 ± 1.5, 19.2 ± 2.8, 59.4 ± 3.5, 82.4 ± 2.7% of damaged metaphases at doses of 10, 20, 40, and 60 mg/kg. Chromatid centric and acentric fragments and exchange-type aberrations represented by acentric and centric rings and chromatid exchanges prevailed in the spectrum of aberrations (figure). Etoposide at doses of 40 and 60 mg/kg induced multiple chromosome violations in 9.6 and 47.2% of oocytes, respectively.

Based on the obtained data, etoposide was used at a dose of 20 mg/kg in further experiments, since the pronounced clastogenic mutagen effect at higher doses complicated the adequate estimation of aneugenic effects.

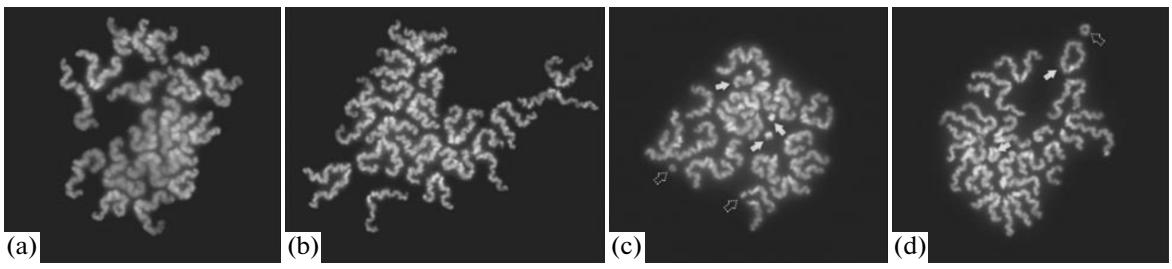
Data on the influence of ACC on aneugenic effects of etoposide are presented in Table 3.

ACC completely prevented the yield of oocytes with hypohaploid and hyperhaploid chromosome sets (induced by etoposide) in 6-week-old female mice (with decreasing indices to the control values). At the same time, the PSSC level did not differ significantly.

ACC completely suppressed PSSC in oocytes (induced by etoposide) in 9-week-old mice. A decrease in the percentage of hypohaploid oocytes was observed; however, the differences were statistically insignificant. In addition, an increase in the yield of oocytes arrested at the MI meiosis stage was detected.

ACC statistically significantly (by 2 times) decreased the yield of hypohaploid oocytes in 12-week-old female mice but did not influence the level of polyploid oocytes. A significant decrease in the percentage of oocytes with delayed meiotic maturation (from 2.7 to 1.1%) was also observed. No significant differences were detected for the PSSC index.

Data on the estimation of the ACC effect on chromosome aberrations in oocytes (induced by etoposide) are presented in Table 4. Etoposide induced violations in 15.6, 22.4, and 35.2% of oocytes in 6-, 9-, and 12-week-old mice, respectively. Only chromatid fragments were observed in the spectrum of chromosome aberrations. ACC statistically significantly decreased the clastogenic effect of etoposide in all age groups. At the same time, the most pronounced protective effect (an 80% decrease) was detected in 9-week-old females as compared to 32 and 52% for 6- and 12-week-old animals, respectively.



Numerical and structural chromosome aberrations in mouse MII oocytes induced by etoposide (magnification ×1000; Hoechst dyeing 33258). (a) metaphase without chromosome violations; (b) metaphase with hypohaploid ($n = 19$) chromosome set; (c) centric (continuous arrows) and acentric (contour arrows) chromatid fragments; (d) centric (continuous arrows) and acentric (contour arrows) ring chromatids.

Table 3. Influence of ACC on numerical chromosome violations in mouse oocytes induced by etoposide

Experimental conditions	Oocytes at meiosis MI stage, %	Metaphases (%) with chromosome number			Polyhaploid metaphases, %	Metaphases with PSSC, %
		$n < 20$	$n = 20$	$n > 20$		
Negative control	0.5	0	100	0	0	0
6-week-old female mice						
Etoposide 20 mg/kg	0	3.9	96.1	0	2.6	1.3
+ ACC 200 mg/kg	0	0**	100	0	0*	1.5
9-week-old female mice						
Etoposide 20 mg/kg	0	6.1	93.9	0	0	5.0
+ ACC 200 mg/kg	2.2	4.4	95.6	0	0	0**
12-week-old female mice						
Etoposide 20 mg/kg	2.7	5.6	94.4	0	2.8	1.4
+ ACC 200 mg/kg	1.1*	2.3**	97.7	0	3.4	0

*, $p < 0.05$; **, $p < 0.01$ as compared with the effect of etoposide.

Table 4. Influence of ACC on structural chromosome aberrations in mouse oocytes induced by etoposide

Experiment conditions	Per 100 cells				Total damaged metaphases ($M \pm m$), %
	chromatid fragments	chromosome fragments	exchanges	cells with MD	
Negative control	0	0	0	0	0
6-week-old female mice					
Etoposide 20 mg/kg	23.4	0	0	0	15.6 ± 2.2
+ ACC 200 mg/kg	10.6	0	0	0	10.6* ± 1.5
9-week-old female mice					
Etoposide 20 mg/kg	28.5	0	0	0	22.4 ± 3.1
+ ACC 200 mg/kg	8.9	0	0	0	4.4** ± 0.6
12-week-old female mice					
Etoposide 20 mg/kg	53.5	0	0	0	35.2 ± 5.0
+ ACC 200 mg/kg	25.0	0	0	0	17.0** ± 2.4

*, $p < 0.05$; **, $p < 0.001$ as compared with the effect of etoposide.

DISCUSSION

The clastogenic effects of etoposide in mouse oocytes were demonstrated for the first time in the work by Mailhes et al. [9]. The mutagen at doses of 20, 40, and 60 mg/kg caused a dose-dependent increase in the yield of cells with chromosome aberrations in 21, 42, and 86% of cells against the background of an absence of oocytes with aberrations in the control animals. Similar results were obtained in the experiments carried out in the present work. Chromatid breaks in the pericentromeric regions and multiple chromosome damage prevailed in the spectrum of aberrations (which is in agreement with data on the localization of topoisomerase II molecules) mainly in the pericentromeric region and along the axis of both arms of the chromosomes [10]. At the same time, the authors failed to provide an objective estimation of aneugenic violations induced by etoposide in the cited work. They registered 21.5% of hypohaploid oocytes in intact mice, which was explained by methodical artifacts. Based on this, the aneugenic effect of etoposide was indirectly estimated according to the ratio of hyperhaploid oocytes in the control and experimental

groups. In the present study, it was reliably established by the applied technique that etoposide has a dose-dependent aneugenic effect in mouse oocytes with induction of the yield of mainly hypohaploid oocytes.

The generation of triple complex DNA-topoisomerase II-etoposide (leading to the inhibition of DNA-ligase activity of topoisomerase II and the origin of double DNA breaks) underlies the mechanism of the clastogenic effect of etoposide [11]. The aneugenic effect of the mutagen is associated with the damage from the process of DNA decatenation (mediated by topoisomerase II) before meiosis anaphase I, which results in nondisjunction of sister chromatids [12]. It was demonstrated by electronic microscopy that violation of the structural organization of the synaptonemic complex at the late stage of pachytene and diplotene occurs under the effect of etoposide (resulting in the generation of univalents of autosomal and sex chromosomes) [13]. It is obvious that the antimutagenic effects of ACC (detected in the present study) cannot be explained by the known mechanisms of its protective effect (antioxidant and/or dismutagenic activity) [5]. Prooxidant activity was demonstrated for etoposide; however, its contribution to the genotoxic effect is insignificant [8]. The detected spectrum of aberrations with a predominance of the chromosome breaks exactly in the region of topoisomerase II localization is in favor of this. In turn, experimental data on the absence of an ACC influence on the antitumor activity of etoposide do not explain the protective effect of ACC due to its dismutagenic activity [6]. In a study by Liu et al. [14], it was established that ACC prevents an age-dependent decrease in telomerase activity and telomere shortening in mouse oocytes. ACC suppressed the inhibiting effect of curcumin on telomerase expression in vitro [15]. At the same time, it was demonstrated that an increase in telomerase expression in tumor cells promotes the origin of a resistance to the cytostatic effect of etoposide in them [16]. Thus, the influence on telomerase activity can be considered one of the possible mechanisms of the protective effect of ACC against the effects of etoposide. Analysis of the literature data also allows the assumption of the indirectness of the modifying effect of ACC by a direct influence on topoisomerase II activity, cell cycle or DNA-repair [5, 6, 17].

It is important to note the depending of the manifestation of etoposide and ACC effects on the age of the experimental animals. The manifestation of cytogenetic effects of etoposide directly depended on the age of mice. An increase in the sensitivity to the effects of mutagens with age was repeatedly demonstrated [1]. At the same time, the anticlastogenic activity of ACC was most pronounced in 9-week-old females (an 80% decrease), while aneugenesis modification (100% decrease) was most pronounced in 6-week-old females. It is difficult to explain the mechanisms of the observed phenomenon within the present study.

Thus, it was demonstrated that ACC has a pronounced antimutagenic activity against cytogenetic effects of etoposide in mouse oocytes. Together with data on antimutagenic effects in male germ cells [8], the data obtained in the present study determine the prospects of further development of ACC as an antimutagenic agent for decreasing the negative effects of etoposide in germ cells. The results of the study are also essential to validate the used technique on the accounting of genotoxic effects in oocytes for the assessment of mutagenic and mutagen-modifying activity.

REFERENCES

1. Durnev, A.D. and Seredenin, S.B., *Mutageny: skrining i farmakologicheskaya profilaktika vozdeistvii* (Mutagens: Screening and Pharmacological Prevention of Effects), Moscow: Meditsina, 1998.
2. Yauk, C.L., Aardema, M.J., Benthem, J., et al., Approaches for identifying germ cell mutagens: report of the 2013 IWGT workshop on germ cell assays, *Mutat. Res.*, 2015, vol. 783, pp. 36–54. doi 10.1016/j.mrgentox.2015.01.008
3. Pligina, K.L., Zhanataev, A.K., Chaika, Z.V., and Durnev, A.D., Method of cytogenetic assay of mouse oocytes, *Bull. Exp. Boil. Med.*, 2013, vol. 156, no. 1, pp. 114–118. doi 10.1007/s10517-013-2292-0
4. Morozova, T. and Andrushchishina, T., Acetylcysteine in clinical practice, *Vrach*, 2007, no. 12, pp. 37–38.
5. De Flora, S., Cesarone, C.F., Balansky, R.M., et al., Chemopreventive properties and mechanisms of *N*-acetylcysteine: the experimental background, *J. Cell Biochem.*, 1995, no. 22, pp. 33–41. doi 10.1002/jcb.240590806
6. De Flora, S., Izzotti, A., Albini, A., et al., Antigenotoxic and cancer preventive mechanisms of *N*-acetyl-L-cysteine, *Cancer Chemoprevention: Promising Cancer Chemoprevention Agents*, Humana Press, 2004, vol. 1, pp. 37–67. doi 10.1007/978-1-59259-767-3_3
7. Travis, L.B., The epidemiology of second primary cancers, *Cancer Epidemiol. Biomarkers Prev.*, 2006, vol. 15, no. 11, pp. 2020–2026. doi 10.1158/1055-9965. EPI-06-0414
8. Attia, S.M., Dominant lethal mutations of topoisomerase II inhibitors etoposide and merbarone in male mice: a mechanistic study, *Arch. Toxicol.*, 2012, vol. 86, no. 5, pp. 725–731. doi 10.1007/s00204-011-0799-6
9. Mailhes, J.B., Marchetti, F., George, L.P., and Barnhill, D.R., Preferential pericentric lesions and aneuploidy induced in mouse oocytes by the topoisomerase II inhibitor etoposide, *Teratog. Carcinog. Mutagen*, 1994, vol. 14, pp. 39–51. doi 10.1002/tcm.1770140106
10. Xiao-Meng Li, Chao Yu, Zhong-Wei Wang, et al., DNA topoisomerase II is dispensable for oocyte meiotic resumption but is essential for meiotic chromosome condensation and separation in mice, *Biol. Reprod.*, 2013, vol. 89(5), no. 118, pp. 1–11. doi 10.1095/biolreprod.113.110692

11. Bromberg, K.D., Burgin, A.B., and Osheroff, N., A two-drug model for etoposide action against human topoisomerase II, *J. Biol. Chem.*, 2003, vol. 278, no. 9, pp. 7406–7412. doi 10.1074/jbc.M212056200

12. Gómez, R., Viera, A., Berenguer, I., et al., Cohesin removal precedes topoisomerase IIα-dependent decatenation at centromeres in male mammalian meiosis II, *Chromosoma*, 2014, vol. 123, nos. 1–2, pp. 129–146. doi 10.1007/s00412-013-0434-9

13. Sukhacheva, T.V., Bogush, T.A., and Kolomiets, O.L., Destructive effect of DNA topoisomerase II inhibitor vepesid on mouse spermatogenesis, *Bull. Exp. Biol. Med.*, 2003, vol. 135, no. 5, pp. 464–469. doi 10.1023/A:1024919510096

14. Liu, J., Liu, M., Ye, X., et al., Delay in oocyte aging in mice by the antioxidant *N*-acetyl-L-cysteine (NAC), *Hum. Reprod.*, 2012, vol. 27, no. 5, pp. 1411–1420. doi 10.1093/humrep/des019

15. Hsin, I.L., Sheu, G.T., Chen, H.H., et al., *N*-Acetyl cysteine mitigates curcumin-mediated telomerase inhibition through rescuing of Sp1 reduction in A549 cells, *Mutat. Res.*, 2010, vol. 688, nos. 1–2, pp. 72–77. doi 10.1016/j.mrfmmm.2010.03.011

16. Sato, N., Mizumoto, K., and Kusumoto, M., Up-regulation of telomerase activity in human pancreatic cancer cells after exposure to etoposide, *Br. J. Cancer*, 2000, vol. 82, no. 11, pp. 1819–1826. doi 10.1054/bjoc.2000.1117

17. Grdina, D.J., Murley, J.S., and Roberts, J.C., Effects of thiols on topoisomerase-II alpha activity and cell cycle progression, *Cell Proliferation*, 1998, vol. 31, nos. 5–6, pp. 217–229. doi 10.1111/j.1365-2184.1998.tb01199.x

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