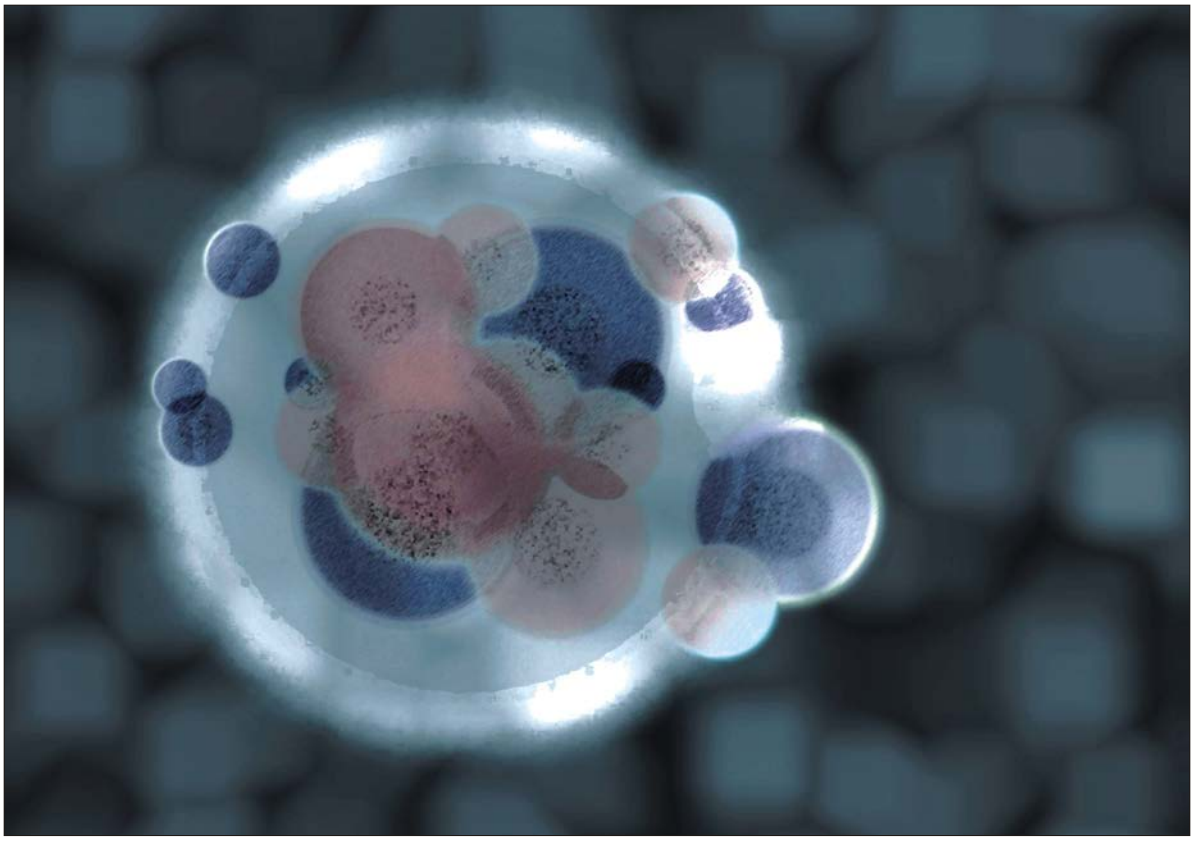

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Cytogenetic Effect of the Trypanocidal Drug Berenil in Blood Cultures of River Buffalo

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Abstract: Berenil (Diminazine aceturate) is an active trypanocidal drug used in farm animals such as cattle and river buffalo. In human, berenil is an effective drug in the treatment of early stage of African trypanosomiasis and cutaneous leishmaniasis. In this work, the cytogenetic effect of berenil was tested in the lymphocyte cultures of river buffalo at three final concentrations; 0.25, 0.50 and 1 mg ml^{-1}. Chromosomal aberrations, sister chromatid exchanges and micronucleus test are the three cytogenetic parameters used in this study. The results demonstrated that the numbers of cells with different types of structural chromosomal aberrations, including breaks, gaps, deletions, fragments and centric fusions were increased significantly in cultures treated with different doses of berenil compared to the control. This increase was dose dependent where there was a positive correlation between increased drug concentration and induction of chromosomal aberrations. The frequency of sister chromatid exchanges and the formation of micronuclei in all lymphocyte cultures treated with different doses of berenil were increased significantly compared to the control and these increases were also dose dependent. In conclusion, the three cytogenetic parameters used to evaluate the effect of berenil revealed that this drug has a strong cytogenetic effect on the river buffalo lymphocytes *in vitro*.

Key words: Berenil, chromosomal aberrations, micronucleus, SCEs

INTRODUCTION

The animal production depends mainly on the general health conditions, which are impaired by several diseases. One of the most important diseases affecting farm animals is Surra disease. This disease is caused by Trypanosoma evansi that seemed to exhibit a well-defined condition as a major world food producing animal health problem. This disease in cattle and buffalo is usually chronic and it is characterized by anemia, progressive loss of condition, emaciation, loss of milk yield, abortion and immunosuppression[1,2].

Berenil (Diminazine aceturate) is an active trypanocidal drug used in horses, dogs, rabbits and chickens as well as cattle, sheep and river buffalo[3-6]. In human, berenil is an effective drug in the treatment of early stage of African trypanosomiasis and cutaneous leishmaniasis[7,8].

The ability of berenil to interfere with activities of eukaryotic topoisomerase II has been reported by Portugal[9] and Boos and Stopper[10]. DNA topoisomerase II is an essential nuclear enzyme involved in the process of DNA replication and chromosome segregation.

In this study, we evaluate the cytogenetic effect of berenil on river buffalo lymphocyte cultures *in vitro*. The formation of chromosomal aberrations, the induction of sister chromatid exchanges and the measurement of micronuclei formations are the three cytogenetic parameters used in this study.

MATERIALS AND METHODS

Cytotoxicity test of the drug: The mutagenic effect of the drug was tested at three final concentrations; 0.25, 0.50 and 1 mg ml^{-1}. These tested doses were chosen according to the cytotoxicity test[11]. This test is established as a standard assay for the detection of chemical compound genotoxicity on mammalian cell cultures *in vitro*. According to the cytotoxicity test procedures, we prepared eight blood lymphocytes cultures. The first was considered as a control. The other seven cultures were treated with different concentrations of the drug, 1, 10, 100, 200, 500 μg, 1 mg and 2 mg ml^{-1}. The mitotic index frequency (the number of dividing cells in 2000 cells) was scored in each one of eight cultures.

The concentration (1 mg ml^{-1}) which reduces the mitotic index to about 50% of the control level, was taken as the higher dose. The half (500 μg ml^{-1}) and the quarter (250 μg ml^{-1}) concentrations of the higher dose were taken as medium and low doses for the cytogenetic analysis.

Culture of blood lymphocytes: Blood samples were taken from healthy animals, which had no treatment during the last 3 months. For chromosomal aberrations and sister chromatid exchanges, one ml of whole heparinized blood was cultured at 38.5°C for 72 h in 5 ml RPMI 1640 medium (Gibco) supplemented with 20% fetal calf serum

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(Gibco), 0.1% penicillin-streptomycin (Gibco), 1% L-glutamine (Gibco) and 4% phytohaemaglutinin (Gibco). The blood cultures for sister chromatid exchanges (SCEs) were treated with bromodeoxyuridine (BrdU, Sigma) at a final concentration of 10 μg ml^{-1}, 24 h after culture initiation[12].

After 24 h from culture initiation, the berenil (Hochest Co.) was added to the cultures at the three final tested concentrations that are 0.25, 0.5 and 1 mg ml^{-1}.

Chromosome preparation: Colchicine (Sigma) at a final concentration of 20 μg ml^{-1} was added to the cultures for chromosomal aberrations and sister chromatid exchanges, 1½ h before harvest. At harvest, the cells were treated with a hypotonic solution (0.075M KCl) for 20 min the cells were fixed three times with fixative (3 methanol : 1 acetic acid). The cells were spread onto cold slides dipped in 70% ethyl alcohol and the slides were prepared by air drying method.

For micronucleus test, the hypotonic treatment was performed with distilled water-medium (4:1) and 2% fetal calf serum for 10 min. The cells were fixed 2 times with fixative (3 methanol:1 acetic acid). The cells were spread onto cold slides dipped in 70% ethyl alcohol and the slides were prepared by air drying method.

Slide staining: For chromosomal aberrations, the slides were stained with 10% Giemsa stain solution (diluted with phosphate buffer, pH 6.8) for 30 min and washed twice in phosphate buffer. The slides were air dried and examined under light microscope. For micronucleus test, the slides were stained with 5% Giemsa stain solution (diluted with phosphate buffer, pH 6.8) for 6 min.

For sister chromatid exchanges, the fluorescence-photolysis-giemsa technique was used[13]. The slides were stained with an aqueous solution of Hoechst 33258 (50 μg ml^{-1}, Sigma) for 20 min and then rinsed in distilled water. On a warmer tray at 50°C, the slides were layered with McIlvaines buffer and subjected to fluorescent black blue light at a distance of 5 cm for 55 min. The slides were stained with 4% Giemsa, diluted with 1 part McIlvaine buffer and 4 parts distilled water, for 5 min.

Scoring: In each animal for different concentration and control, 100 metaphases were examined for chromosomal aberrations, whereas 30 metaphases in the second division were analyzed for SCEs. One thousands cells were examined in each animal for measuring micronucleus formation.

Statistical analysis: Chi-square test (2×2 contingency table) was used for the chromosomal aberrations data analysis, while the t-test was used to evaluate sister chromatid exchanges and micronucleus formation induced by this drug.

RESULTS

Analysis of chromosomal aberrations: The frequency of structural chromosomal aberrations was recorded in blood cultures of river buffalo treated with berenil at three tested concentrations (Table 1). 500 well-spread metaphases for each drug concentration as well as for control were screened. The chromosomal aberrations screened in this study involved breaks, gaps, deletions, fragments and centric fusion. The cells have more than one type of aberrations were recorded under each type.

In treated cultures with low dose (0.25 mg ml^{-1}) the numbers of cells with breaks and gaps were increased significantly at levels $p<0.001$ and $p<0.01$, respectively. The percentage of cells with deletions and fragments was 7.6% compared to 4.2% in control. The numbers of cells with deletions and fragments and centric fusion were increased significantly at $p<0.05$.

At the medium dose (0.5 mg ml^{-1}) the numbers of cells with breaks and gaps increased at highly significant level $p<0.001$ compared to the control, while the significant level of the increases in the number of cells with deletions and fragments was $p<0.01$. The number of cells with centric fusion was 19 cells, which increased at significant level $p<0.01$ compared to the control.

At the highest drug concentration (1 mg ml^{-1}). The number of cells with all types of structural chromosomal aberrations; breaks, gaps, deletions and fragments and centric fusion were increased at highly significant level $p<0.001$.

The results showed that the numbers of total aberrant cells with structural chromosomal aberrations (including or excluding gaps) increased at highly significant level $p<0.001$ in the three tested concentrations compared to the control. The only exception was the number of total aberrant cells with structural aberration excluding gap, which was increased at the significant level $p<0.01$. Comparing the numbers of cells with one aberration as well as the number of cells with more than one aberration between controls and the three tested concentrations revealed that these numbers were increased at significant levels $p<0.01$ and $p<0.001$ in the three tested concentrations (Table 1).

Sister chromatid exchanges: The effect of berenil on the frequency of sister chromatid exchanges was shown in Table 2. 150 metaphases in second division were examined for each drug concentration as well as for

Table 1: Frequency of chromosomal aberrations induced by berenil in river buffalo lymphocytes *in vitro*

Drug conc.	No. of animals	No. of examined cells	No. of cells with structural chromosomal aberration: Breaks No.	Breaks %	Gaps No.	Gaps %	Deletions and fragments No.	Deletions and fragments %	Centric fusion No.	Centric fusion %
Control	5	500	34	6.8	29	5.8	21	4.2	4	0.8
0.25 mg ml^{-1}	5	500	67***	13.4	56**	11.2	38*	7.6	13*	2.6
0.5 mg ml^{-1}	5	500	93***	18.6	82***	16.4	45**	9.0	19**	3.8
1 mg ml^{-1}	5	500	139***	27.8	116***	23.2	70***	14.0	26***	5.2

Table 1: Continued

Drug conc.	No. of animals	No. of examined cells	No. of total aberrant cells: With gap No.	With gap %	Without gap No.	Without gap %	No. of cells with one aberration No.	No. of cells with one aberration %	No. of cells with more than one aberrations No.	No. of cells with more than one aberrations %
Control	5	500	61	12.2	32	6.4	57	11.4	4	0.8
0.25 mg ml^{-1}	5	500	118***	23.6	62**	12.4	92**	18.4	26***	5.2
0.5 mg ml^{-1}	5	500	161***	32.2	79***	15.8	119***	23.8	42***	8.4
1 mg ml^{-1}	5	500	217***	43.4	101***	20.2	143***	28.6	74***	14.8

* p<0.05, ** p<0.01, *** p<0.001, N.B. The cells have more than one type of aberrations were recorded repeatedly under each type

Table 2: Effect of berenil on sister chromatid exchanges in lymphocyte cultures of river buffalo

Drug concentration	No. of animals	No. of examined cells	No. of SCEs	SCEs / cell: Range	SCEs / cell: Mean±SD
Control	5	150	767	3-8	6.5±1.18
0.25 mg ml^{-1}	5	150	1280	5-13	8.53±1.62*
0.5 mg ml^{-1}	5	150	1605	8-12	10.7±1.27***
1 mg ml^{-1}	5	150	1740	9-14	11.6±1.72***

* p<0.05 *** p<0.001

Table 3: Effect of berenil on frequencies of binucleated cells and micronuclei (MN)

Drug concentration	No. of animals	No. of examined cells	Binucleated cells No.	Binucleated cells Mean±SD	Binucleated cells with MN No.	Binucleated cells with MN Mean±SD	Micronuclei (MN) No.	Micronuclei (MN) Mean±SD
Control	5	5000	621	124.2±9.68	48	9.6±2.14	51	10.2±2.56
0.25 mg ml^{-1}	5	5000	542	108.4±6.37*	79	15.8±2.33**	84	16.8±2.93**
0.5 mg ml^{-1}	5	5000	499	99.8±5.71**	156	31.2±4.17***	174	34.8±4.96***
1 mg ml^{-1}	5	5000	464	92.8±3.43***	182	36.4±4.63***	228	45.6±5.12***

* p<0.05 ** p<0.01 *** p<0.001

control. The means of SCEs/cell±SD in the three tested concentrations were 8.53±1.62, 10.7±1.27 and 11.6±1.72, respectively compared to 6.5±1.18 in the control cultures. By using the t-test for the analysis of SCEs data, we found that this drug increased the frequency of sister chromatid exchanges at all three tested concentrations. These frequencies increased significantly at p<0.05 in cultures treated with low dose and at p<0.001 in cultures treated with both medium and high doses.

Micronuclei formation: For each drug concentration as well as for control, 5000 cells were examined for measuring micronuclei formation. The micronuclei were scored in binucleated cells. The numbers and means±SD of binucleated cells, binucleated cells with MN and micronuclei were recorded at each drug concentration and control (Table 3). The t-test was used to analysis the data of micronuclei formation. The results showed that the numbers of binucleated cells were decreased at the significant level p<0.05 with low dose, at p<0.01 with medium dose and at a highly significant level p<0.001 with high dose. The numbers of binucleated cells with MN as well as the numbers of micronuclei were increased at significant level p<0.01 in cultures treated with low dose and at highly significant level p<0.001 in cultures treated with both medium and high doses.

DISCUSSION

Berenil is an effective trypanocidal drug used in the treatment of trypanosoma in different animals including farm animals[3-6] and also in human[7,8]. Berenil is recognized as an agent that binds to nucleic acid duplex. The generally accepted mode of berenil binding is via complexation into the minor groove of AT-rich dominas of DNA double helices[14,15]. This affinity of berenil binding to higher ordered nucleic acid structures and complexation with a DNA and RNA triple helix was reported in many studies[16-20].

The cytogenetic effect of berenil was evaluated in this work at three final concentrations 0.25, 0.5 and 1 mg ml^{-1}, using river buffalo lymphocyte cultures.

The formation of chromosomal aberrations, the induction of sister chromatid exchanges and the measurement of micronuclei formations are the three cytogenetic parameters used in this study.

Our results revealed that the numbers of cells with different types of structural chromosomal aberrations; including breaks, gaps, deletions, fragments and centric fusions were increased significantly with the three tested doses of berenil compared to the controls (Table 1). The percentages of total aberrant cells (with and without gaps) showed increases in low dose (23.6 and 12.4%) medium dose (32.2 and 15.8%) and high dose (43.4 and 20.2%) compared to the control (12.2 and 6.4%). These increases were dose dependent where there was a positive correlation between drug concentration and induction of structural aberrations.

The mutagenic effect of this drug was also illustrated by the increase in the number of cells with one aberration and the number of cells with more than one aberration in low dose (18.4 and 5.2%) medium dose (23.8 and 8.4%) and high dose (28.6 and 14.8%) compared to the control (Table 1).

The induction of structural chromosomal aberrations investigated in this study agrees with the reports recorded the induction of different fragile sites in lymphocyte cultures treated with berenil[21-26].

The high level of chromosomal aberrations induced by berenil might be related to the ability of this drug to interfere with activities of eukaryotic topoisomerase II[9]. The DNA topoisomerase II is an essential nuclear enzyme that modulates DNA topology during multiple cellular processes such as DNA replication and chromosome segregation. There are different steps in the action of topoisomerase II, all of which are potential targets for inhibition through drugs and also for cellular and genetic toxicity as well as for mutagenesis[10].

The other two cytogenetic parameters studied in this work were sister chromatid exchanges and micronuclei formation. SCEs have been established as a cyto-diagnostic tools to assay the genetic damages induced by mutagens and carcinogens in the population[27-30]. Our results showed that berenil increased the frequencies of SCEs at a significant level $p<0.05$ in lymphocyte cultures treated with low dose. In cultures treated with both medium and high drug concentrations, the berenil increased the frequency of SCEs at highly significant level $p<0.001$ (Table 2). The elevation of the SCE frequency by berenil investigated in this study agreed with the previous reports by Schmid *et al.*[31] and Tsuji *et al.*[26] in human culture lymphocytes.

The micronucleus test is widely employed in different areas in biological monitoring. It has become a tool to evaluate the mutagenic effect of drugs before they are commercialized[32-34]. The results of this study showed that the numbers of binucleated cells were decreased to 108.4±6.37 in low dose, 99.8±5.71 in medium dose and 92.8±3.43 in high dose compared to 124.2±9.68 in the control (Table 3). This result revealed that this drug has a cytotoxic effect on the number of cell divisions. On the other hand, the numbers of binucleated cells with MN and the frequency of micronuclei were increased at significant level $p<0.01$ in cultures treated with low dose and at $p<0.001$ in cultures treated with both medium and high doses. The same effect of berenil on the induction of micronuclei in mouse lymphoma cells was reported by Boos and Stopper [20].

Based on our results, the three cytogenetic parameters used to evaluate the effect of diminazine aceturate (berenil) revealed that this drug has a mutagenic effect on the river buffalo lymphocyte cultures.

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