EFFECT OF CHRONIC ALCOHOL CONSUMPTION ON APOPTOSIS OF LYMPHOCYTES: ROLE OF CASPASE-3 AND FAS LIGANDS

N. Boyadjieva and G. Bocheva

Department of Pharmacology and Toxicology, Medical Faculty,
Medical University – Sofia

Summary. Ethanol has been shown to acutely increase the apoptosis in various cells. But the effect of ethanol on FasL-mediated apoptosis of lymphocytes is not well established. The aim of present study is to evaluate the mitogen-induced proliferation in vitro and apoptosis of lymphocytes obtained from the spleens of alcohol and nonalcohol fed rats. We also determined whether caspase-3 or soluble FasL play a role in apoptotic effect of ethanol on lymphocytes. Here, we found that chronic alcohol administration increased both apoptosis and caspase-3 activity and decreased the number of T lymphocytes via depressing the proliferation and activating the apoptotic cell death in cultured splenocytes. Furthermore, we identified that caspase-3 and soluble FasL play a role in apoptosis of lymphocytes.

Key words: *apoptosis, Fas, Fas ligand (FasL), alcohol, caspase-3, T cells*

INTRODUCTION

Apoptosis is a step of the cell cycle, important in the regulation of immune cell populations, which is essential mechanism used to remove activated T cells. Ethanol (EtOH) intake affects both innate and adaptive immune responses. Furthermore, several studies have shown that chronic ethanol intake in both humans and mice is associated with changes of different subsets of cytotoxic T-lymphocytes (e.g., CD8+ T cells) [15, 16, 24, 27, 31]. Excessive alcohol consumption has a suppressive effect on the immune system's ability to clear virus-infected cells and cells that have undergone neoplastic transformation. Previously, it has been demonstrated that chronic alcohol consumption decreased the total number of lymphocytes as well as the number of various subsets of T cells of male

rats [3]. The mechanism of ethanol's effect on lymphocytes is not well established. Moreover, the role of apoptosis in lymphocyte pathways of death is not clear.

It is well documented that Fas is involved in both T-cell receptor- and membrane Ig-induced apoptosis [21, 25]. Binding of the Fas ligand (FasL) to the Fas receptor (CD95), a member of TNF receptor family, may be an important element in apoptosis of lymphocytes. FasL not only induces apoptosis in Fas receptor-bearing target cells, but it is also able to transmit signals into the FasL-expressing cell via its intracellular domain [17]. The effect of ethanol on FasL-mediated apoptosis of lymphocytes is also not well established.

The aim of present study is to evaluate the mitogen-induced proliferation in vitro and apoptosis of lymphocytes obtained from the spleens of alcohol and nonalcohol fed rats. We also determined whether caspase-3 or soluble FasL play a role in apoptotic effect of ethanol on lymphocytes.

MATERIALS AND METHODS

Animals

Male rats (180-200 g) were used for acute and chronic experiments. Rats were divided into 2 groups – controls and ethanol treated group (5 animals in each group). **Acute studies:** Male rats were treated orally with alcohol (4 g/kg; 20% solution; acute ethanol treatment-Ea) for a period of 6 h. Then rats were sacrificed, spleens were taken and splenocytes were isolated. Splenocytes were cultured for a period of 24 or 48 h, and studies on cell proliferation, apoptosis or Fas ligands were performed. **Chronic studies:** Male rats were alcohol-fed with an alcohol diet for a period of 3 weeks. Isolated splenocytes were cultured for further studies.

Study of Lymphocyte Proliferation

Lymphocytes were isolated from the spleens of male rats treated with 4 g/kg 20% solution of ethanol for a period of 6 h or 3 weeks. Splenocytes were isolated in accordance with previously published method [2] and cultured (1 x10 (3) cells per well) in duplicate in 96-well culture plates in complete growth medium containing RPMI1640, 1% penicillin/streptomycin, 2% glutamine, and 10% untreated fetal bovine serum. Cells were treated with or without concanavalin A (Con A; 5 μg/ml) or phytohemagglutinin (PHA; 5 μg/ml; all from Sigma Chemical Co., St. Louis, MO) for 48 h. The number of cells was determined by a cell viability assay, which uses a tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) [9]. Briefly, the cells were incubated with 1 mg/ml (100 μl per well) of MTT for 4 h at 37°C. The MTT solution was removed, and 100 μl of 2-propanol was added into each culture well for 30 min. The developing color was measured with a spectrophotometer (HTS 7000, PerkinElmer, Boston, MA) with a 560-mm outer diameter. The number of lymphocytes was determined by using a standard curve of untreated spleenocytes (2.5, 10, 20, 40, 80, and 160 000) from ad libitum–fed

rats and the linear regression method. Although this cell viability assay is used to determine the cell number, it does not indicate whether an increase of cell number is due to increased cell proliferation or decreased cell death.

Apoptosis ELISA

The apoptosis in cultured splenocytes was measured by Nucleosome ELISA assay in nucleosome units/ml by following the instructions of manufactures (Calbiochem, USA).

Caspase-3 activity

The Caspase-3 activity assay is a fluorometric, immunosorbent enzyme assay for the specific, quantitative *in vitro* determination of caspase-3 activity in microplates (Oncogene Research Product, Boston, USA). Caspase-3 activity was determined in pmol/min by following the instructions of manufactures.

Fas Ligand ELISA

The Fas Ligand (FasL) was determined by FasL ELISA (Oncogene Research Products, Boston, USA). It is a "sandwich" enzyme immunoassay employing mouse monoclonal antibodies. It is achieved quantitative by the construction of a standard curve using known concentrations of FasL. The FasL were determined in ng/ml by following the instruction of manufactures.

Statistics

The mean and SE of the data were determined and are presented in the text and figures. Data were analyzed with ANOVA. The differences between groups were determined with the Student-Newman-Keuls test. A value of $p < 0.05$ was considered a significant difference.

RESULTS

By using both splenocytes and polymorph nuclear cells in primary cultures and performing MTT assay, we have previously shown that long-term exposure to ethanol decreased mitogen-activated proliferations of lymphocytes [3]. In this study, we used cells from the spleens of male rats, and found also that the chronic ethanol exposure decreased the concanavalin A- and phytohemagglutinin-activated proliferation of cultured splenocytes (Fig. 1A and 1B; Fig. 2A and 2B). The results showed that alcohol decreased mitogen-induced proliferation of lymphocytes. The acute exposure to ethanol did not affect the proliferation of cells. We also found that chronic ethanol intake increased the amount of DNA damage in the cells as determined by the nucleosome activity (a marker for DNA damage in the cells; Fig. 3) [1]. Taken together, the results demonstrate that ethanol depresses the proliferation of splenocytes and causes apoptotic cell death.

Studies were conducted to examine whether ethanol-induced cell apoptosis in splenocytes involves an activation of caspase-3. This enzyme is known to increase endonuclease activity and cause cell apoptosis [6]. In our chronic

study, ethanol increased the activity of caspase-3 in cells taken from spleens of alcohol-fed male rats (Fig. 5). The data shown in Fig. 4 indicate that ethanol increases the levels of soluble FasL in cultured splenocytes in a dose-dependent manner.

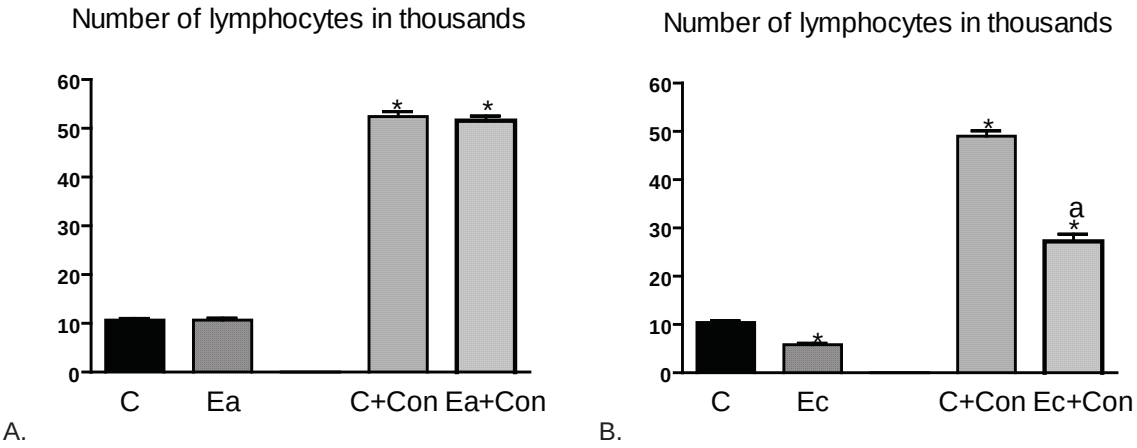

Fig. 1. Chronic alcohol consumption inhibits the Concanavalin A-activated (Con) proliferation of cultured splenocytes. Male rats were treated with alcohol (4 g/kg; 20% solution; per os: acute ethanol treatment-Ea; section A) or alcohol-fed for a period of 3 weeks (Ec; section B). The splenocytes were isolated, cultured with or without Con (5 μg/ml) for a period of 48 h. The number of cells was determined by MTT assay. Data are mean+ SE from 5 observations. *p< 0.001 as compared with controls without Con; a/p< 0.001 as compared with controls + Con

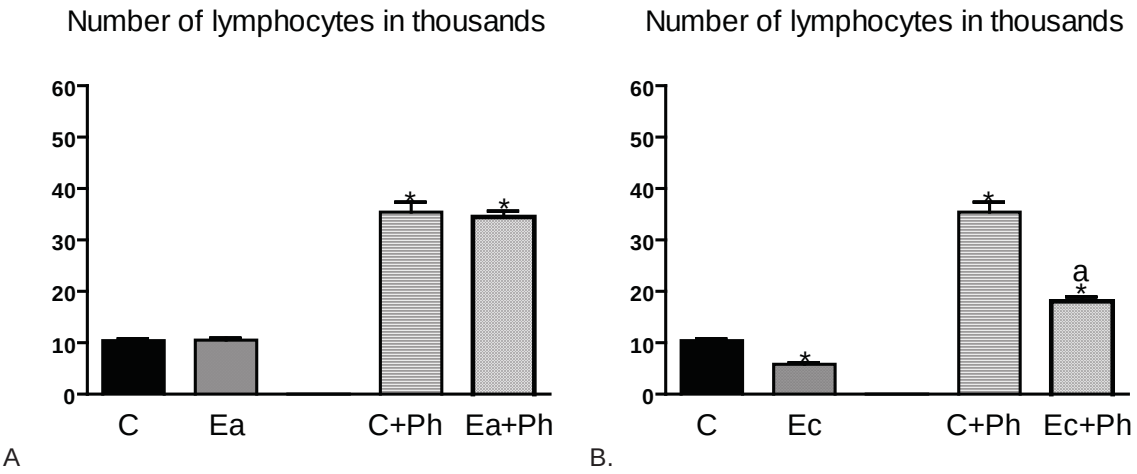

Fig. 2. Effect of ethanol on phytohemagglutinin-stimulated (Ph) proliferation of cultured splenocytes. Male rats were treated with alcohol (4 g/kg; 20% solution; per os: acute ethanol treatment-Ea; section A) or alcohol-fed for a period of 3 weeks (Ec; section B). The splenocytes were isolated, cultured with or without Ph (5 μg/ml) for a period of 48 h. The number of cells was determined by MTT assay. Data are mean+ SE from 5 observations. *p < 0.001 as compared with controls without Ph; a/p < 0.001 as compared with controls +Ph

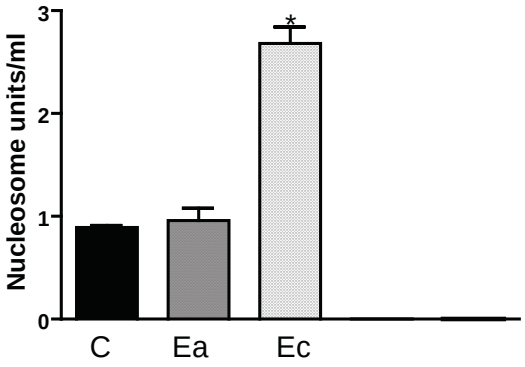

Fig. 3. Chronic alcohol consumption stimulates apoptosis in cultured splenocytes. Male rats were treated with alcohol (4 g/kg; 20% solution; per os: acute ethanol treatment-Ea) or alcohol-fed for a period of 3 weeks (Ec). The splenocytes were isolated, cultured for a period of 48 h. Then cells were lysed and apoptosis was determined by nucleosome ELISA assay in nucleosome units/ml. Data are mean+ SE from 5 observations. *p < 0.001

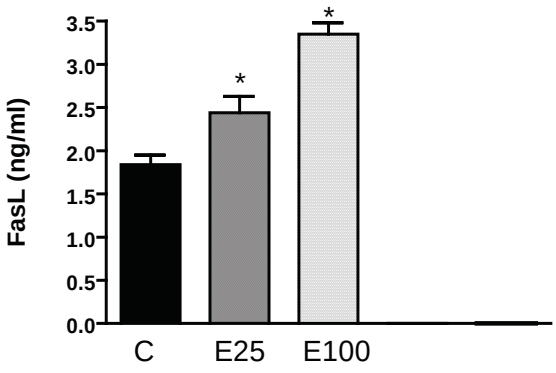

Fig. 4. Acute effects of alcohol on Fas ligands (FasL) in splenocytes. The splenocytes were isolated from control male rats, cultured for a period of 48 h. Then cells were treated with various doses of ethanol (50 mM or 100 mM) for 48 h. Cells were lysed and soluble FasL were determined by ELISA assay in ng/ml. Data are mean+ SE from 5 observations. *p < 0.001

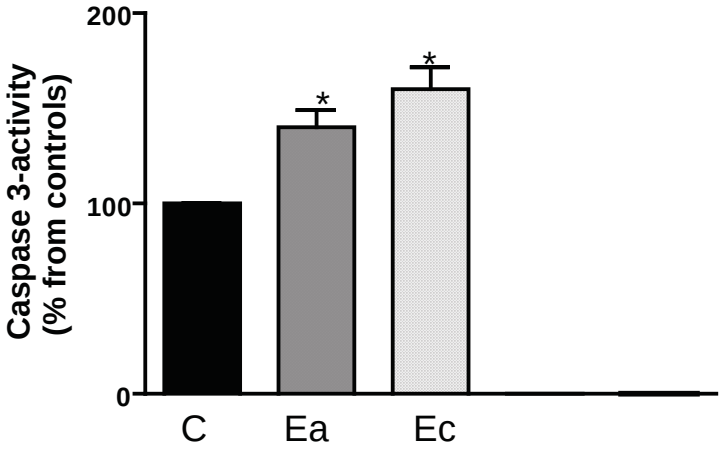

Fig. 5. Acute and chronic effects of ethanol on caspase-3 activity in cultured splenocytes. Male rats were treated with alcohol (4 g/kg; 20% solution; per os: acute ethanol treatment-Ea) or alcohol-fed for a period of 3 weeks (Ec). The splenocytes were isolated, cultured for a period of 48 h. Then cells were lysed and caspase-3 activity was determined by ELISA. The results are present as percentage of controls. Data are mean+ SE from 5 observations. *p < 0.001

DISSCUSSION

The data presented here provide evidence that chronic ethanol administration decreases the concanavalin A- and phytohemagglutinin-activated proliferation

of cultured splenocytes from male rats. We further show that ethanol exposure increases apoptosis in cells. Furthermore, ethanol exposure activates caspase -3 and increases the cellular levels of FasL of cells.

Ethanol has been shown to acutely increase the apoptosis in various cells [7, 10]. Ewald SJ and Shao H (1993) demonstrated that ethanol increases apoptotic cell death of thymocytes in vitro [11]. It was also published that ethanol promotes T cell apoptosis through the mitochondrial pathway [12]. Various studies suggest that apoptosis plays a role in control of immune function [13, 14, 19, 20, 23]. Using fetal hypothalamic cells in primary cultures and DNA fragmentation assay, we have previously shown that long-term exposure to ethanol induced apoptotic death of hypothalamic cells during the developmental period [8]. Moreover, chronic alcohol consumption decreased either, the total number of lymphocytes and the number of various subsets of T cells [3], as well as both number and activity of NK cell in rats [3-5]. The results presented here demonstrated that splenocytes died via apoptotic mechanism. Taken together, the data suggest that alcohol decreased the number of T lymphocytes via depressing the proliferation and activating the apoptotic cell death.

The Fas/FasL is one of the best-studied death systems. Fas-mediated apoptosis is known as one of mechanisms by which activated T cells undergo cell death [14]. The sensitivity of T cells toward Fas-mediated apoptosis can be modulated by the level of FasL expression upon activation. Like other TNF family members, FasL is a homotrimetric molecule. Each FasL trimer binds three Fas molecules and makes a complex which triggers a cascade of subcellular changes. This cascade involves a number of proteins. It makes a “death domain” and contains enzymes as caspases, which play an important role in apoptosis. We have previously shown the role of caspase-3, 8 and 9 in apoptosis of lymphocytes. It is well established that caspases have a central role in the regulation of most types of apoptotic pathways in various cells. Activation of the caspase cascade results in the cleavage of cellular substrates that induces the biochemical as well as morphological features of apoptosis.

Furthermore, the caspase-3 locates in the downstream of the caspase cascade and functions as an executor of cells. Because activated T cells express FasL, and Fas-mediated signaling plays important parts in the induction of lymphocyte apoptosis [18, 30], the contribution of FasL interaction in the apoptotic effect of alcohol was studied. The data demonstrated that ethanol in a dose dependent manner increases the levels of FasL in cultured splenocytes. There was not a significant change in FasL in splenocytes of rats exposed chronically to ethanol. It remains to be established whether soluble FasL could have systematically consequences by increasing apoptosis in various tissue as well as in spleen. It was also demonstrated, that soluble FasL showed either agonist or antagonistic activity in various activating mechanisms.

In the present study, exposure of male rats to chronic alcohol increased both apoptosis and caspase-3 activity in cultured splenocytes. The increased activ-

ity of caspase-3 in cells suggestsing that alcohol facilitates apoptosis of splenocytes through promoting caspase activation with possible Fas-dependent pathway. These data suggest the possibility that chronic ethanol exposure induces apoptosis in lymphocytes by involvement of caspase-3 and FasL cascade. However, further studies need to be conducted to determine the mechanism by which ethanol is affecting FasL in lymphocytes.

REFERENCES

1. A l l e n, R. T., W. J. Hunter 3rd et D. K. Agrawal. Morphological and biological characterization and analysis of apoptosis. – J. Pharmacol. Toxicol. Methods, **37**, 1997, 215-228.
2. B o y a d j i e v a, N. I. et al. Opioid antagonist naltrexone disrupts feedback interaction between mu and delta opioid receptors in splenocytes to prevent alcohol inhibition of NK cell function. – J. Immunol., **173**, 2004, № 1, 42-49.
3. B o y a d j i e v a, N. I. M. et al. Beta-endorphin modulation of lymphocyte proliferation: effects of ethanol. – Alcohol Clin. Exp. Res., **26**, 2002, № 11, 1719-1727.
4. B o y a d j i e v a, N. et al. Chronic ethanol inhibits NK cell cytolytic activity: role of opioid peptide beta-endorphin. – J. Immunol., **167**, 2001, № 10, 5645-5652.
5. B o y a d j i e v a, N., G. Meadows et D. Sarkar. Effects of ethanol consumption on beta-endorphin levels and natural killer cell activity in rats. – Ann. N. Y. Acad. Sci., **885**, 1999, 383-386.
6. C o h e n, G. M. Caspases: the executioners of apoptosis. – Biochem. J., **326**, 1997, № 1, 1-16.
7. C h e n, C. P. et al. Ethanol induces apoptotic death of developing beta-endorphin neurons via suppression of cyclic adenosine monophosphate production and activation of transforming growth factor-beta1-linked apoptotic signaling. – Mol. Pharmacol., **69**, 2006, № 3, 706-717.
8. D e, A. et al. Cyclic AMP and ethanol interact to control apoptosis and differentiation in hypothalamic beta-endorphin neurons. – J. Biol. Chem., **269**, 1994, № 43, 26697-26705.
9. D e n i z o t, F. et R. Lang. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. – J. Immunol. Methods, **89**, 1986, № 2, 271-277.
10. D i a m o n d, I. et A. S. Gordon. Cellular and molecular neuroscience of alcoholism. – Physiol. Rev., **77**, 1997, 1-20.
11. E w a l d, S. J. et H. Shao. Ethanol increases apoptotic cell death of thymocytes in vitro. – Alcohol Clin. Exp. Res., **17**, 1993; 359-365.
12. K a p a s i, A. A. et al. Ethanol promotes T cell apoptosis through the mitochondrial pathway. – Immunology, **108**, 2003, 313-320.
13. K a t z, G. G. et al. Signaling for ethanol-induced apoptosis and repair in vitro. – Clin. Biochem., **34**, 2001, № 3, 219-227.
14. K r a m m e r, P. H., R. Arnold et I. N. Lavrik. Life and death in peripheral T cells. – Nat. Rev. Immunol., **7**, 2007, 532-542.
15. L a s o, F. J. et al. Abnormalities of peripheral blood T lymphocytes and natural killer cells in alcoholic hepatitis persist after a 3-month withdrawal period. – Alcohol Clin. Exp. Res., **21**, 1997, № 4, 672-676.
16. L a s o, F. J. J. et al. Chronic alcohol consumption is associated with an increased cytotoxic profile of circulating lymphocytes that may be related with the development of liver injury. – Alcoholism: Clin. Exp. Res., **34**, 2010, 876–885.
17. L ü c k e r a t h, K. et al. Immune modulation by Fas ligand reverse signaling: lymphocyte proliferation is attenuated by the intracellular Fas ligand domain. – Blood, **117**, 2011, № 2, 519-529.

18. M c C o n k e y, D. J. et al. Calcium activated DNA fragmentation kills immature lymphocytes. – FASEB J., **3**, 1989, 1843-1849.
19. M u r p h y, K. M., A. B. Heimberger et D. Y. Loh. Induction by antigen of intrathymic apoptosis of CD4+ and CD8+ TCR thymocytes in vivo. – Science, **250**, 1990, 1720-1722.
20. N e u m a n, M. G. Apoptosis in diseases of the liver. – Crit. Rev. Clin. Lab. Sci., **38**, 2001, № 2, 109-166.
21. O r b a c h, A. et al. CD40·FasL and CTLA-4·FasL fusion proteins induce apoptosis in malignant cell lines by dual signaling. – Am. J. Pathol., **177**, 2010, № 6, 3159-3168.
22. P a n, H. N. et al. Chronic ethanol consumption inhibits hepatic natural killer cell activity and accelerates murine cytomegalovirus-induced hepatitis. – Alcohol Clin. Exp. Res., **30**, 2006, 1615-1623.
23. P e r n e y, P. et al. Specific alteration of peripheral cytotoxic cell perforin expression in alcoholic patients: a possible role in alcohol-related diseases. – Alcohol Clin. Exp. Res., **27**, 2003, 1825-1830.
24. S a n t o s - P e r e z, J. L. et al. T-cell activation, expression of adhesion molecules and response to ethanol in alcoholic cirrhosis. – Immunol. Lett., **50**, 1996, N 3, 179-183.
25. S c h ü t z, C. et al. Alloantigen specific deletion of primary human T cells by Fas ligand (CD95L)-transduced monocyte-derived killer-dendritic cells. – Immunology, **133**, 2011, № 1, 115-122.
26. S i n g h a l, P. C. et al. Ethanol-induced macrophage apoptosis: the role of TGF-β. – J. Immunol., **162**, 1999, 3031-3036.
27. S o n g, K. et al. Chronic ethanol consumption by mice results in activated splenic T cells. – J. Leucoc. Biol., **72**, 2002, № 6, 1109-1116.
28. S t r a s s e r, A., M. Pellegrini. T-lymphocyte death during shutdown of an immune response. – Trends Immunol., **25**, 2004, 610-615.
29. S z a b o, G. et P. Mandrekar. A recent perspective on alcohol, immunity, and host defense. – Alcohol Clin. Exp. Res., **33**, 2009, № 2, 220-232.
30. W a t a n a b e – F u k u n a g a, R. et al. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. – Nature, **356**, 1992, 314-317.
31. Z h a n g, H., G. G. et G. G. Meadows. Chronic alcohol consumption in mice increases the proportion of peripheral memory T cells by homeostatic proliferation. – J. Leukoc. Biol., **78**, 2005, 1070-1080.

✉ *Address for corespondence:*
N. Boyadjieva
Department of Pharmacology and Toxicology
Medical Faculty
Medical University
2 Zdrave str.
1431 Sofia
✆ 91-72-61