Investigation of nuclear DNA content and cell cycle phases in rat liver cells under chlorpromazine administration

Rykalo N. A.¹, Baylo O. V.²
¹Medical University of Innsbruck, Innsbruck, Austria
²Pirogov Vinnytsia National Medical University, Vinnytsia, Ukraine

ARTICLE INFO
Received: 25 April 2023
Accepted: 31 May 2023

UDC: 577.213.3:616.36-03.93:615.214

CORRESPONDING AUTHOR
e-mail: bayloov@gmail.com
Baylo O. V.

CONFLICT OF INTEREST
The authors have no conflicts of interest to declare.

FUNDING
Not applicable.

Introduction
The liver is a unique unpaired organ that performs more than a dozen vital functions around the clock, one of which is the metabolism of xenobiotics. The vast majority of drugs are metabolized by liver cells, so it is difficult to overestimate the importance of this organ. Hepatotoxicity of antipsychotic drugs remains an urgent problem of modern medicine, and concerns many branches of medicine, including psychiatry, not only pharmacology and hepatology [1, 16, 21]. This is due to the fact that most of the syndromes and diseases encountered by psychiatrists in their daily practice, such as bipolar disorders or schizophrenia, require long-term, sometimes life-long medication correction and support [3, 11]. Most of the drugs used in psychiatric practice are hepatotoxic [8, 12]. Chlorpromazine as an antipsychotic, neuroleptic, sedative, muscle relaxant, and antiemetic continues to be used both in Ukraine [10, 11] and around the world, despite its side effects [4, 22]. However, the mechanisms of the toxic effect on the liver and other organs and tissues have not been fully elucidated and are still being investigated [4].

It is an interesting scientific fact that patients with schizophrenia who receive treatment with antipsychotic drugs with an antitumor effect, such as Chlorpromazine, have a lower incidence of cancer [9]. That is, on the one hand, hepatotoxicity is a significant side effect of the drug, which can limit the spectrum of its use and cohort of patients for treatment, on the other hand, it can be a promising drug for use in oncology practice [13, 20].

The purpose of the work is to investigate the content of nuclear DNA and the cell cycle phase of rat liver cells under the conditions of administration of Chlorpromazine in doses from 3.5 mg/kg to 28 mg/kg for 30 and 60 days.
Materials and methods
The study was conducted on 60 sexually mature female rats. All experiments were conducted in accordance with the "General Ethical Principles for Animal Experiments" approved by the First National Congress on Bioethics (Kyiv, 2001) and harmonized with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes" (Strasbourg, 1986). The bioethics committee of the National Pirogov Memorial Medical University, Vinnytsia approved that the work was performed in compliance with ethical principles (protocol No. 8 dated 22.10.2020). Before the start of the experiment, the animals were kept for 14 days in a vivarium under quarantine conditions. The drug Chlorpromazine (trade name - "Aminazine", dose - 25 mg/ml) produced by PrJSC "Halychpharm" (Arterium) was used for the study.

The experimental animals were divided evenly into 12 groups (group 1 was the control group, groups from 2 to 12 were experimental). The hepatotoxicity of Chlorpromazine was studied for 30 and 60 days, depending on the dose of the drug.

Chlorpromazine was administered intragastrically with a metal probe with oil once a day at a dose of 3.5 mg/kg, 7 mg/kg, 14 mg/kg, 21 mg/kg, and 28 mg/kg. The calculation of doses of chlorpromazine was carried out according to the constant of biological activity according to the recommendations of Yu. R. Rybolovlev and R. S. Rybolovlev [18]. The control group (group 1) included intact animals. Animals from groups 2 to 6 (average initial body weight was 128.2±17.4 g) received Chlorpromazine for 30 days in a dose: animals of group 2 - 3.5 mg/kg, group 3 - 7 mg/kg, group 4 - 14 mg/kg, group 5 - 21 mg/kg, 6 group - 28 mg/kg. Experimental animals from groups 6 to 11 (average initial body weight was 125.0±18.7 g) for 60 days received Chlorpromazine in a dose: animals of group 7 - 3.5 mg/kg, group 8 - 7 mg/kg, group 9 - 14 mg/kg, 10 group - 21 mg/kg, 11 group - 28 mg/kg. It should be noted that during the experiment all animals of 11 groups died.

After completion of the 30- and 60-day experiment, all rats were subjected to euthanasia by intraperitoneal injection of Thiopental solution at the rate of 0.1 mg/g followed by decapitation. At the end of each part of the experiment, blood was collected from all rats for further biochemical research.

Materials and methods used to determine the DNA content (cell cycle, DNA fragmentation) in the nuclei of liver cells in rats. DNA content in the nuclei of rat liver cells was determined by DNA flow cytometry.

Nucleus suspensions from rat liver cells were obtained using a ready-made solution for the study of nuclear DNA CysStain DNA Step 1 from Partec, Germany, according to the manufacturer's protocol-instructions. This solution allows extraction of nuclei and labeling of nuclear DNA with diamidino-phenylindole (DAPI). In the process of manufacturing nuclear suspensions, disposable CellTrics 50 μm filters (Partec, Germany) were used.

Flow analysis was performed on a multifunctional research flow cytometer "Partec PAS" by Partec, Germany. UV radiation was used to excite DAPI fluorescence. From each sample of nuclear suspension, 20,000 events were registered. Events (cell nuclei) with a DNA content of <4c were subject to analysis.

Cyclic analysis of cells was performed using FloMax software (Partec, Germany) in full digital correspondence according to a mathematical model, where:

- G0G1 - percentage ratio of G0G1 phase cells to all cells of the cell cycle (DNA content = 2c);
- S is the percentage ratio of cells in the phase of DNA synthesis to all cells of the cell cycle (DNA content=2c and <4c);
- G2+M - percentage ratio of the G2+M phase to all cells of the cell cycle (DNA = 4c, or polyploid);
- Determination of DNA fragmentation (apoptosis) is performed by highlighting the SUB-G0G1 area on DNA histograms - RN2 before the G0G1 peak, which indicates cell nuclei with DNA content<2c.

Statistical processing of the obtained results was carried out in the license package "Statistica 6.0" using non-parametric estimation methods. The nature of the distributions for each of the variation series was evaluated, the average values for each characteristic and the standard square deviation were determined. The reliability of the difference in values between independent quantitative indicators was determined using the Mann-Whitney U-test.

Results
When analyzing the G0G1 indicator - percentage ratio of cells in the G0G1 phase (with diploid DNA content) to all cells of the cell cycle of rat liver tissue (Fig. 1), a dose-dependent effect of Chlorpromazine on this indicator is observed both in animals that were administered the drug for 30 and 60 days. Thus, in the liver cells of rats, when Chlorpromazine was administered for 60 days at a dose of 21 mg/kg, the G0G1 interval increased by 21.5 % (p<0.05 compared to the control), 14 mg/kg - by 14.8 % (p<0.05), 7 mg/kg - by 12.0 % (p<0.05) and 3.5 mg/kg - by 13 % (p<0.05). When animals were administered Chlorpromazine at the same dose (14 mg/kg), but for different durations, the G0G1 interval increased by 11.1 % during the 60-day experiment, compared to the 30-day experiment (p<0.05). In animals administered the drug for 30 days, a significant increase in the percentage of diploid nuclei was registered in the 5 (by 15.3 %, p<0.05) and 6 group (by 20.4 %, p<0.05).

S phase research - percentage ratio of cells in the phase of DNA synthesis to all cells of the cell cycle (DNA content >2c and <4c) showed an increase in this indicator in all animals under the condition of drug administration (Fig. 2), which, in our opinion, indicates the activation of the cell cycle in response for alteration. Significant changes, compared to the control, were registered in animals of all groups that received Chlorpromazine for 60 days (in group 7 - by 48.2 %, 8 - 89.9 %, 9 - 74.0 %, 10 - 92.9 %, p<0.05). When the drug was administered for 30 days, there was a significant
difference in animals of 5 (increase by 44.4 %, p<0.05) and 6 group (by 33.0 %, p<0.05) differed significantly from the control compared to animals of the 1 group.

Determination of DNA fragmentation (apoptosis) showed, that in animals of all groups, with the exception of the 2nd group (which received Chlorpromazine at a dose of 3.5 mg/kg daily for 30 days), the SUB-G0G1 interval was statistically different from the intact animals of the control group (Fig. 4). In particular, when Chlorpromazine was administered at a dose of 7 to 28 mg/kg for 30 days, nuclear DNA fragmentation significantly increased, compared to both intact animals and a group of animals that received Chlorpromazine at a dose of 3.5 mg/kg, which suggests apoptosis of hepatocytes. When Chlorpromazine was administered at a dose of 7 mg/kg, SUB-G0G1 significantly increased by 68.2 % (p<0.05 compared with intact rats and p<0.05 compared with animals that received Chlorpromazine at a dose of 3.5 mg/kg), at 14 mg/kg - at 72.7 % (p<0.05 and p<0.05, respectively), 21 mg/kg - significantly more than 1.9 times (p<0.05 and p<0.05, respectively). The highest level of apoptosis was in animals of the 6th group that received Chlorpromazine at a dose of 28 mg/kg, as the fragmentation index increased by 2.1
times \((p<0.05\) and \(p<0.05\), respectively, compared to intact animals and a dose of 3.5 mg/kg), and also by 22.9 % in comparison with animals that received Chlorpromazine at a dose of 14 mg/kg. When Chlorpromazine was administered for 60 days, apoptosis increased by 74.8 % in animals of group 7, 2.1 times in group 8, 2.3 times in group 9, and 2.7 times in animals in group 10. In our opinion, it should be noted that when the same doses of Chlorpromazine were administered for 30 and 60 days, nuclear DNA fragmentation was higher when the drug was administered for a longer period of time. Thus, in animals of group 7, the percentage of fragmented nuclei was higher by 70.9 % \((p<0.05)\) compared to animals of group 2, between groups 8 and 3 the difference was 25.0 % \((p<0.05)\), 9 and 4 - 34.6 % \((p<0.05)\), 5 and 10 - 40.7 % \((p<0.05)\).

Discussion

The data of the statistical analysis of the results obtained by us indicate a direct dependence of the effect of Chlorpromazine on the nuclear DNA of rat liver tissue cells on the dose and duration of administration. The higher the dose and, especially, the duration of drug administration, the more intensive changes in the phases of the cell cycle are registered in animals. Thus, in animals 5 (Chlorpromazine was administered at the rate of 21 mg/kg for 30 days) and 6 groups (28 mg/kg for 30 days), all the studied indicators (G0G1, S, G2M and SUB-G0G1) had a statistically significant difference with animals control group. When the drug was administered for 60 days to animals of all groups (7-10), from the minimum to the maximum dose (3.5 to 21 mg/kg), all the studied parameters were statistically significantly different from the inactive animals of the control group. It should be added that all animals that received Chlorpromazine at a dose of 28 mg/kg for 60 days died during the experiment.

According to the results of the data we obtained, using the flow cytometry method, namely measuring the percentage of hepatocyte DNA with a diploid, tetra-, polyploid and subdiploid DNA set (which is a sign of the death of liver cells by apoptosis [6, 19]), a clear dose-dependent effect of Chlorpromazine was established, as well as an increase in the hepatotoxicity of the studied drug during long-term administration. The data we obtained do not contradict the literature, according to which no toxic effect was detected when chlorpromazine was administered to mice at a dose of 3 mg/kg [5].

Our previously published data [2] regarding the morphological changes in liver tissue upon administration of Chlorpromazine in different doses and durations are significantly supplemented and logically explained by this study of nuclear DNA content and cell cycle phases of rat liver cells under the conditions of Chlorpromazine administration.

In our opinion, the index of nuclear DNA fragmentation, which induces apoptosis [6], which is determined by isolating SUB-G0G1, i.e. cell nuclei with DNA content <2c, deserves special attention.

According to the results of our research, apoptosis of hepatocytes significantly increased when Chlorpromazine was administered for 30 days at a dose of 7, 14, 21, and 28 mg/kg, and when the drug was used for 60 days at a dose of 3.5-21 mg/kg. The obtained data correspond to the data of the literature [7, 8, 15], according to which Chlorpromazine, together with tyrosine kinase inhibitors, increases the apoptosis of tumor cells. Chlorpromazine also acts as a destabilizer of lysosomal membranes, which also promotes apoptosis [17]. On the other hand, [22] indicate that Chlorpromazine inhibits the mitochondrial pathway of apoptosis due to increased expression of tissue factor. Also, mechanisms of increased apoptosis during chlorpromazine therapy include increased autophagy by inhibiting the phosphatidylinositol 3-kinase Akt/mTOR pathway in human U-87MG glioma cells [20].

According to the authors, this proapoptotic therapeutic potential of Chlorpromazine can be used in complex therapy of various types of neoplasms [5, 7, 14, 23].

According to A. J. Jou and co-authors [8] Chlorpromazine enhances the proliferation block precisely in the G2/M phase due to the regulation of the PI3K/AKT/mTOR-mediated autophagy mechanism in cancer.

Prospects for further development, in our opinion, can be a scientifically based review of the indications and contraindications for the appointment of Chlorpromazine, based on the results of experimental studies, namely the antitumor properties of this drug.

Conclusions

1. Chlorpromazine has a dose-dependent hepatotoxic effect: increasing its dose to experimental animals from 7 to 28 mg/kg significantly increases the percentage of nuclear DNA fragmentation in liver tissue, which is a sign of hepatocyte death by apoptosis. Chlorpromazine at a dose of 3.5 mg/kg did not increase the process of apoptosis of hepatocytes, while at a dose of 21 mg/kg and 28 mg/kg it showed the maximum effect of hepatotoxicity, increasing the level of apoptosis by 1.9 and 2.1 times, respectively.

2. With long-term use of Chlorpromazine, the hepatotoxic effect manifested in increased fragmentation of hepatocyte nuclear DNA increases, which, in our opinion, should be taken into account when treating patients.

References


Investigation of nuclear DNA content and cell cycle phases in rat liver cells under chlorpromazine administration


дозах від 3,5 мг/кг до 28 мг/кг впродовж 30 та 60 днів. Дослідження проведено на 60 статевозрілих щурах-самицях. Хлорпромазин вводили один раз на добу щоденно впродовж 30 та 60 дб у дозі 3,5 мг/кг, 7 мг/кг, 14 мг/кг, 21 мг/кг та 28 мг/кг. Вміст ДНК в ядрах клітин печінки щурів визначали методом проточної ДНК-цитометрії. Циклічний аналіз клітин виконували засобами програмного забезпечення Flomax (Partec, Німеччина), де визначали: відсоток ядер в інтервалі G0G1 клітинного циклу, у фазі S, інтервалі G2M, а також показника апоптозу - SUB-G0G1 ділянки на ДНК-гістограмах. Статистична обробка отриманих результатів проведена за допомогою U-критерія Мана-Уітні. Результати проведеного дослідження показали, що хлорпромазин має дозозалежний гепатотоксичний ефект: при збільшенні дози введення даного препарату у щурів від 7 до 28 мг/кг достовірно збільшується відсоток фрагментованих ядер в тканині печінки, що є ознакою загибелі гепатоцитів шляхом апоптоза. Встановлено, що хлорпромазин в дозі 3,5 мг/кг не посилює апоптоз гепатоцитів, тоді як у дозі 21 та 28 мг/кг препарат виявив найбільшу гепатотоксичність, збільшуючи рівень апоптозу у 1,9 та 2,1 (р<0,05) рази відповідно. При застосуванні хлорпромазину впродовж 60 днів гепатотоксичний ефект посилюється, що проявляється у достовірному збільшенні фрагментізації ядерної ДНК гепатоцитів, що, на нашу думку, варто враховувати при проведенні тривалої терапії пацієнтам.
Ключові слова: хлорпромазин, апоптоз, фази клітинного циклу, печінка.