A technology of experimental studies on the xenobiotic element sorption characteristics of representatives of the intestinal normal flora

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ABSTRACT
Modern views on the general level of intoxication of an organism by heavy metals are based solely on the extent of effects on organs and organ systems of macroorganisms while the impacts on representatives of the normal flora and their role in the organism’s mechanisms of detoxification as a whole are not taken into account. We proposed technology of experimental studies based on a complex variety of methods. The technology allows estimating the degree of effects of xenobiotic elements on representatives of the intestinal normal flora and their sorption characteristics, which is an important factor in the assessment of mechanisms of detoxification in oral intoxication. The obtained experimental data indicate high sorption characteristics of lead cations in the studied microorganisms, which, in our view, is primarily associated with microorganisms’ mechanisms of detoxification. The study found that the most pronounced bioaccumulative characteristics for lead were registered in *Escherichia coli* (65% of the total volume of the elements introduced in the substrate) and, in the case of cadmium, – *Enterococcus faecium* (33.2%). The minimal accumulation scores were revealed for *Lactobacillus acidophilus* (42.3% and 5.3%, respectively).

Keywords: technology of sorption characteristic assessment, normal flora, microbiota, xenobiotics, heavy metals.

1. INTRODUCTION
Nowadays, only cadmium, lead, mercury, and antimony are unequivocally considered as toxic elements. Activities of other numerous heavy metals in biological systems vary. It is true that there are no harmful substances – there are harmful concentrations [1].

The role of lead ions as a biogenic element is not great; it is known that lead participates in the metabolic processes of the bone tissue. In the environment, lead is widespread as a result of natural processes, including erosion, forest fires, the decay of radon, and as a result of human activities – waste from ore extraction, burning leaded fuel [2, 3, 4].

But there is a group of metals which vital necessity has not been revealed. They include cadmium [5, 6]. It is known that cadmium is an active antagonist of several essential elements such as copper and zinc. Mutual antagonism between these elements is a consequence of their isomorphic substitution in biological systems. Namely, increased consumption of one of these elements can cause deficiency of another owing to the substitution of it in some functional sites of binding [7, 8, 9].

Some metal ions are essential for microorganisms. When viewing the patterns of interaction of heavy metals with microorganisms, one should also take into account the toxic effects of heavy metal ions on microorganisms [10]. Metal accumulation by microorganism cells is of two-phase nature. The initial phase is not dependent on the energy status of the cell and is caused by sorption of metal by components of the cell wall [11].

Summarizing the above, we set the aim to develop a technology of xenobiotic sorption characteristic evaluation of normal flora representatives in the intestine.

2. MATERIALS AND METHODS
As a xenobiotic factor, we used salts of lead and cadmium with high levels of dissociation in aqueous solutions. Applying these salts enables to create maximal concentrations of cations of the elements in a nutritious substrate. Selected and identified representatives of the normal flora of the intestine served as the research objects.

To achieve our aim, we implemented the following methods of research:

1. The method of microorganism selection and identification. The necessity of applying this method lies in the fact that the intestinal microbiota is represented by a variety of microorganisms. Toward this end, we extracted a fragment of intact rat intestines in compliance with the rules of asepsis for selection of representatives of the optionally anaerobic normal flora. One gram of that content was introduced in 10 ml of the physiological solution, and then it was thoroughly pipetted with subsequent transferring one ml into the next test tube with the same amount of the isotonic solution. Only eight shifts were made (this was necessary for the maximal dilution with a view to determining the number of colony-forming units (CFU)). The contents of the tubes in the volume of 50 μl were transferred into sterile Petri dishes filled with 1.5% beef-extract agar followed by incubation at the
research, we had previously prepared sterile 100 ml bottles, a sterile liquid nutritional medium (MSR), daily culture of the studied microorganisms and sterile solutions of metals (in accordance with the technique described above). Experimental studies were conducted in ten replications.

Isolated strains were incubated in the liquid nutrient medium using the batch method of cultivation. Four bottles were filled with MSR agar, a suspension of microorganisms in the concentration of 0.5 by McFarland (McFarland standard), and the working concentration of xenobiotic elements composed of salts with a high level of dissociation in aqueous solutions (the first concentration with no sub-inhibiting effect). Like in the serial dilutions method, we utilized three control samples allowing the qualitative assessment of the research; in case of deviations in the control samples, the results were not taken into account.

The colorimeter CFC-2 was used for the analysis. The mean values of the series of measurements were considered as the result of the analysis. The measurement of the optical density of the bacterial suspension was carried out with an interval of three hours starting with a background measurement. The duration of the study was determined by the time of the beginning of the M-phase of growth concentration in the studied strains as evidenced by the presence of three relatively close values of the relative optical density of the suspension in different time intervals. During the period of incubation, the microorganisms were at the thermostat at 37 °C set on the shaker for constant stirring.

4. Atomic absorption spectrophotometry. The use of this technique was the final stage of our study. The method is based on the property of atoms formed when ash solutions are sprayed in acetylene-air flame to absorb light of a specific wavelength. As atomic absorption spectrophotometer (AASP), there was used the device AAS-1 (Germany) with a set of spectral lamps.

For the preparation of the samples, the sterile bottles were filled with 400 ml of sterile MSR, working concentrations of heavy metals and 4 ml of a suspension of the studied microorganism followed by incubation at 37 °C till the stationary phase of growth in batch culture. After the cultivation, the content of bottles was poured into sterile test tubes and centrifuged for 30 minutes at 3000 rpm. The supernatant was separated from the biomass with an automatic pipette, the biomass of the studied microorganisms was lysed with 5% solution of KOH and further held in a water bath at 96 °C for 20 minutes (for the complete destruction of cells). Both the biomass and the supernatant were exposed to the analytical study for the purposes of determining concentrations of metals in both samples. The presence of metals in the biomass of cells indicates the sorption characteristics of the studied microorganisms. The mean values of ten series of experiments were converted into percentages in the form of concentrations. A loss in the biomass and the supernatant was determined by calculating the difference between the values of the injected concentration of the metal and the amount of its content in the biomass and the supernatant.

5. All the data were subjected to statistical processing by calculating the means and the errors of the mean, one-way
3. RESULTS

During the preliminary study aimed at selecting and identifying representatives of the intestinal microbiota of lab rats, we selected four microorganisms that accounted maximal concentrations of CFU: *Lactobacillus acidophilus*, *Escherichia coli*, *Enterobacter cloacae*, and *Enterococcus faecium*.

Studies of evaluating the resistance of the studied strains of microorganisms to xenobiotic factors (table 1) revealed a general trend of relative resistance to cations of lead (from 0.005 to 0.00125 mmol/ml) and pronounced sensitivity to cadmium (from 0.0003 to 0.0001 mmol/ml).

From the presented in the table data, it should be concluded that the most resistant to lead strains are *Escherichia coli* and *Enterococcus faecium* (sub-inhibiting concentration of 0.005 mmol/ml), while the level of sustainability to cadmium had values 0.0003 and 0.0001 mmol/ml, respectively. *Lactobacillus acidophilus* and *Enterobacter cloacae* have lower values of resistance to lead (0.0025 and 0.00125 mmol/ml) and similar to the other two strains’ values of sensitivity to cadmium (0.0003 and 0.0001 mmol/ml).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Salts</th>
<th>Concentration, M/l</th>
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<tr>
<td></td>
<td>Pb(NO₃)₂</td>
<td>-</td>
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<tr>
<td></td>
<td>CdSO₄*8H₂O</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
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<td></td>
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<td></td>
<td>-</td>
<td>+</td>
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<tr>
<td><em>Enterococcus faecium</em></td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>Pb(NO₃)₂</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CdSO₄*8H₂O</td>
<td>+</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>-</td>
<td>±</td>
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</table>

Figure 1. Influence of Pb(NO₃)₂ on representatives of the optionally anaerobic microbiota of rats’ intestines.

Figure 2. Photos of microorganisms without metals and with lead. Scanning atomic force microscopy. a1, b1, c1 — without metal; a2, b2, c2 — in the presence of lead salts (Peshkov et al., 2015).

Figure 3. Influence of CdSO₄ on representatives of the optionally anaerobic microbiota of rats’ intestines.

Figure 4. Accumulation of lead ions from the nutrient substrate in representatives of the rats’ optionally anaerobic intestinal microbiota.
The next phase of our research aims to assess the extent of influence of the studied heavy metals on growth of the studied microorganisms and the time of the beginning of the M-phase of growth concentrations (Figures 1, 3). The process of metal accumulation occurs in the stationary growth phase, this is due to the fact that this phase includes substrate depletion and accumulation of toxic products forcing bacteria to search for alternative sources of energy and detoxification of the environment.

The data from Figure 1 show a pronounced degree of influence of lead on growth of microorganisms while the values of the relative optical density of the experimental groups were significantly lower than the control ones, which, we believe, is associated with a high concentration of introducing a lead salt with a high level of dissociation in aqueous solutions and cation accumulation on the surface, which, in turn, affects the values of relative optical density. It was recorded that the presence of lead in a nutritious substrate had no significant effect on the time of the beginning of the stationary phase. The analysis of the data presented in Figure 3 shows a pronounced inhibiting action of cadmium cations on the growth of the studied microorganisms. The final phase of the study was to assess the sorption characteristics of representatives of the optionally anaerobic normal flora in rats’ intestines (Figures 4, 5). Presented in the figures data showed the expressed sorption characteristics of the studied microorganisms with respect to cations of the studied metals. Lead is the most actively extracted from the substrate at the surface of a bacterium from 65% (Escherichia coli) to 42.3% (Lactobacillus acidophilus), which correlates with data obtained in previous experiments. The sorption level of cadmium cations (Figure 5) had lower values from 33.2% (Enterococcus faecium) to 5.3% (Lactobacillus acidophilus).

4. CONCLUSIONS

The conducted experimental studies indicate high sorption characteristics of the studied microorganisms. It should be noted that the level of sorption characteristics is of individual (species) nature and depends on biochemical features of the species, in our view.

The technology of evaluation of biotoxicity and accumulative characteristics of microorganisms can be used not only for studying the mechanisms of detoxification, but also for designing and synthesis of new antibacterial compounds based on essential elements of pathogenic microorganisms, which currently has great practical significance at seeking for alternative methods of chemotherapy of infectious diseases taking into account the minimization of negative effects on the normal flora of the organism of humans and animals, as well as for elemental status biocorrection.

5. REFERENCES

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