Original Article

Comparative and Combined *In Vitro* Vasotoxicity of Nanoparticles Containing Lead and Cadmium

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Abstract

In vitro toxicological experiments were performed on an endothelial cell line exposed to different doses of spherical nanoparticles of cadmium and/or of lead sulfides with mean diameter 37 ± 5 nm and 24 ± 4 nm, respectively. Toxic effects were estimated by Luminescent Cell Viability Assay, endothelin-1 concentration and cell size determination. Some dose-response relationships were typically monotonic (well approximated with hyperbolic function) while others were bi- or even 3-phasic and could be described within the expanded hormesis paradigm. The combined toxicity type variated depending on the effect it was assessed by.

Keywords

lead, cadmium, nanoparticles, cell cultures, combined toxic action

Introduction

A comparative toxicological assessment of particulates is being an important subject experimental study within the framework of particle toxicology as a research area in its own right including toxicology of chemically similar metal-containing particles falling within different size ranges, for instance, nanometric particles compared to micrometric ones. At the same time, however, toxic effects of particles of *similar* size but *different* chemical composition have been compared much less despite their growing abundance industrially and environmentally, particularly in the nanometric range in the form of nanomaterials and engineered nanoparticles (NP) and as an important fraction of spontaneous workplace and ambient air aerosols. The latter arise from various metallurgical, welding and chemical technologies[1-4] and often comprise several toxic elements rather than just one metal.

That is why, the effects of various NP combinations were the focus of a range of comparative experimental and mathematical modeling studies carried out by our research team, including the nanotoxicology of Ni and Mn[5,6]; Cu, Zn, Pb[7]; Al, Ti and Si,[8] and Pb and Cd[9] combined in various proportions. Pikula et al., 2020[10] experimented with CdS and ZnS combination.

Alongside this line of research, our aspiration has been to advance the general theory of combined toxicity ([11-16] and many others). In these studies, various acute, subchronic or chronic intoxications were modeled by exposing rats to repeated administration of dissolved salts or of nanoparticles of different toxic elements in different combinations. The health effects thus induced were assessed by a wide range of functional, biochemical and morphometric criteria, and the results could be summarized as follows: "effect additivity" and "dose additivity" generally recognized as 2 opposite paradigms of combined toxicology theory are, in fact, interchangeable. Therefore we assumed that these paradigms depend on which particular method is chosen for describing experimental data

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mathematically rather than on essential differences in fundamental mechanisms of combined toxic action. Moreover, we maintain that the main types of combined toxic action, i.e. additivity, superadditivity (synergism), subadditivity (hidden antagonism) and opposite action (explicit antagonism) are not inherent invariable characteristics of a specific mixture. Indeed, even under exposure to one and the same simple binary combination, these types are revealed depending on the toxic effect considered, the level of this effect, the ratio of the doses involved, and on the presence, if any, of a third, concomitantly acting toxic agent.

Different Cd-Pb combinations are most often present in workplace and ambient air in technologies associated with copper smelting and refining. Both lead and cadmium are toxic for the organism in practically all chemical forms and, thus, are of special research interest. One of the first combinations we assessed for toxicity in a subchronic experiment on rats was one of Cd and Pb in their ionic forms.[17] The data from that experiment were then processed with the help of the Response Surface Method (RSM),[18] which garnered additional support to the above theoretical postulates. This experimental study had been prompted by the results of an epidemiological one[19] that assessed the combined nephrotoxic effect of these 2 metals.

Some time ago we decided to assess experimentally the combined vaso-cardiotoxic effects of lead and cadmium considering the part they both play in human cardiovascular disease. All experimental toxicological studies published thus far involved water solutions of lead and cadmium salts while epidemiological studies considered exposure to lead and/or cadmium without specifying their physical form. In the foregoing, the occupational and environmental hazards of metal-containing nanoparticles for humans were mentioned as important in practical terms, and so the toxic properties of lead and, to a lesser extent, cadmium have been considered in a number of experimental studies, particularly by our team. [7,9,20,21] However, little attention (if any) has been given just to the vaso-cardiotoxicity of such nanoparticles.

As far as we know, the *in vitro* cardiotoxicity of lead and cadmium has been studied only by our team in experiments on HL-1 cardiomyocytes.[9] Judging by decreased adenosine triphosphate–dependent luminescence, cadmium sulfide nanoparticles (CdS-NP) produced a considerably greater cytotoxic effect than lead sulfide ones (PbS-NP). However, in the same dose range, CdS-NP caused fewer calcium spikes whereas PbS-NP produced a similar effect at lower doses. Also, while some doses of CdS-NP and PbS-NP provoked cell hypertrophy, doses causing cardiomyocyte size reduction were also identified. These 3 outcomes were fit either by monotonic dose–response curves well approximated by a hyperbolic function or by non-monotonic ones in different variants. For the latter we derived adequate mathematical expressions by modifying certain models of hormesis available in the literature.

Data analysis with the help of a response surface linear model with a cross-term provided fresh support to the above postulate that one and the same pair of toxic agents may display a diversity of combined action types.

The present paper focuses on the *vasotoxicity* of nanoparticles containing lead and cadmium, alone or in combination.

Materials and Methods

For the experimental modeling of different nano-intoxications, sufficiently stable suspensions of nanoparticles were produced by laser ablation of respective 99.99% pure targets (in this research, of lead sulfide or cadmium sulfide) under a layer of deionized water. This technique is regularly employed by our team and has been described in sufficient detail more than once (e.g. Katsnelson et al.,[1]). In this way, we have obtained spherical NPs of cadmium and lead sulfides with diameter 37 ± 5 nm and 24 ± 4 nm, respectively)

The *in vitro* toxicological experiments were performed on the KST stable cell line from the collection of cell cultures of the Russian Academy of Agricultural Sciences (BioloT Ltd., Saint-Petersburg, Russia). It is a monolayer culture of endothelial cells obtain from bovine embryo coronary vessels. The number of viable cells in the culture was determined with the help of the cell counter LUNA-II (Logos Biosystems, Korea). Prior to performing an experiment, a 20 µL sample was taken from the cell suspension and mixed with an equivalent volume of trypan blue. The resulting suspension was transferred to a disposable slide for counting cells in the instrument. Culture viability was found to be around 80%.

For exposure to nanoparticles, the cells were seeded in a 96-well plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland), 100,000 cells per well in 100 µL nutrient medium. These plates were kept for 24 hours before adding NPs at 37ºC under an atmosphere of 5% CO_2 in a DMEM medium with L-glutamine, 1 g/L glucose, 10% fetal bovine serum (FBS) and 0.5% gentamicin antibiotic. The final NP concentrations in the incubation medium (mcg/mL) with concurrently exposed samples were as follows: CdS-NP 3.75-5-7.5; PbS-NP 7.5-11-15; CdS-NP 3.75 + PbS-NP 7.5; CdS-NP 7.5 + PbS-NP 15; CdS-NP 5 + PbS-NP 15; CdS-NP 7.5 + PbS-NP 7.5; CdS-NP 5 + PbS-NP 11 µg/mL. All exposure variants were performed in quadruplicates. Cell incubation in the NP-containing medium lasted 24 hours.

CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used according to the manufacturer's instruction. The plates were equilibrated to room temperature for approx. 30 minutes and the reconstituted CellTiter-Glo Reagent was added 1:1 to the amount of cell culture medium present in each well. The plates were shaken for 2 min and incubated for additional 10 min at RT before transferring well contents to a luminescence compatible 96-well plate, and luminescence was read on LM-01 T with Kilia software (Immunotech, Beckman Coulter Company, Praha, Czech Republic). The measurement results were expressed in relative luminescence units (RLU).

ET-1 was determined quantitatively in cell lysate using a diagnostic test system by Biomedica (Vienna, Austria). The method is based on enzyme-linked immunosorbent assay. The results were measured in the Epoch Microplate Spectrophotometer (BioTek, USA) at a wavelength of 450 nm and expressed in fmol/ml per 100,000 cells. Cells lysate was obtained by freezing-thawing cell destruction.

For cell size determination, cells were removed from the plate, put on a glass slide and imaged in an optical microscope, 3D Cell Explorer (Nanolive, Switzerland). ImageJ 1.48v image processing program (Wayne Rasband, National Institutes of Health, USA) was used to measure cell and cell-nucleus area, reading the results in μm^2.

Data Mathematical Treatment and Analysis

The experimental data demonstrated an explicitly non-monotonic dose-response dependence. Moreover, within the dose ranges used, this dependence is bi- or tri-phasic for some of the indices. This required approximating it by more complex expressions than the usual models of hormesis (see (Panov et al. 2020[9] and articles cited therein). Since polyphase functions describe a complex and multifactorial biological phenomenon, it would be unlikely to expect that such a dose-response dependence could be adequately expressed by simple functions. For instance, in[9] this dependence is represented by power functions depending on 8 parameters.

In the present paper, a different approximation approach was employed, in which the complexity of the dose-response relationship is hidden within the approximation functions while the form of such approximation itself is very simple: it is a linear combination of the chosen functions. In particular, the template used for approximating the multiphase dose-response relationship was given by the sum:

$$y = b_0 + b_1 J_k(x) + b_2 J_{k+1}(x) + b_3 J_{k+2}(x),$$

where $J_k(x)$ is a Bessel function of the first kind of integer order k (Abramowitz and Stegun: Handbook of Mathematical Functions). Generally, it is sufficient to assume that k = 1, 2, or 3. The parameters b_0, b_1, b_2, b_3 are found by the least-squares method using experimental data.

Note that the proposed form of dose-response approximation is effective exactly where a polyphase hormesis is present. On the contrary, for classical hormesis or a usual monotonic dose-response relationship it would be sufficient to apply simple analytical expressions as used below.

As well as in our previous works on combined toxicity,[5,7,8,11,13,18,12,22] the mathematical modeling of Cd-Pb combined action was performed by the Response Surface Methodology (RSM).

The regression equation describing the response surface $Y = Y (x_1, x_2)$ in our case is:

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2 \tag{1}$$

where Y is a toxicity index, x_1 and x_2 are the doses of the agents participating in the combination (cadmium and lead, respectively). This equation was constructed by fitting the coefficients b_0, b_1, b_2 and b_{12} to experimental data using the usual least squares method. It is inferred that 2 agents produce a unidirectional effect on response Y if both one-way response functions $Y(x_1, 0)$ and $Y (0, x_2)$ either increase or decrease with an increase in x_1 or x_2; on the contrary, 2 agents are assumed to be acting contra-directionally if one function increases while
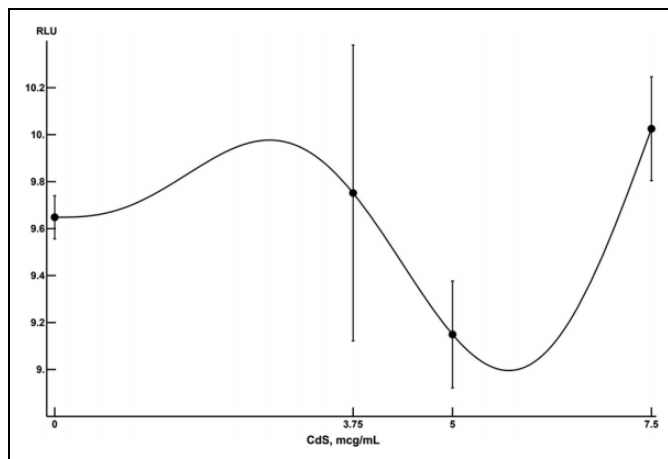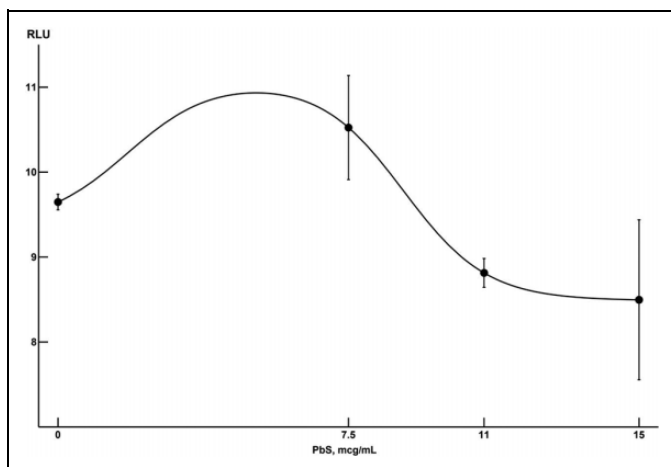

Figure 1. Reduction in ATP-dependent luminescence in endothelium cell culture under exposure to various doses of CdS-NP. The abscissa plots the acting concentrations of nanoparticles in the incubation medium, mcg/mL; the ordinate plots the values of the Relative Light Units (RLU) index in the exposed or control groups. The dots indicate the mean values with the standard error of the mean. The equation for the approximating curve is $y = 9.64833 + 3.94974 J_3(x)$ $8.73696 J_4(x) + 5.65988 J_5(x)$.

the other decreases. According to the response surface approach, even in the case of 2-level agents the equation (1) enables one to predict the magnitude of response y for any combination of agent doses within the experimental range for each of them (rather than at 2 points only). Quasi-sectioning the response surface on different levels, corresponding to different meanings of the outcome **Y**, provides a family of Loewe isoboles which may have the same or different shapes, as well as unidirectional or contra-directional slopes, and thus render the identification of the type of binary combined toxicity both easy and demonstrative.
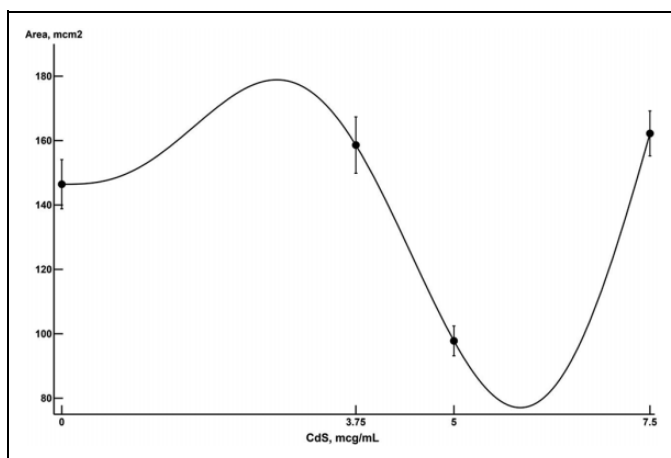
Results and Discussion

Like in the study mentioned in the Introduction,[9] performed with the same NP species on an HL-1 culture of cardiomyocytes, the dose-response function for the culture of endothelial cells often displayed a non-monotonic character. The reader is referred to the above publication for the references to works from which we derived the mathematical models used as such or modified by us for satisfactory approximation of this kind of dose-response relationships. In the same recent publication, we discussed specially the understanding of such non-monotonic dose-response functions within the hormesis paradigm in the broad sense of the term. In the current paper, therefore, we do not repeat this discussion, and confine ourselves to graphic illustration of the relevant results and their model approximation, as well as brief comments relating to the meaning of specific responses.

Figures 1 and 2 display a rather similar pattern of diminishing culture viability as the dosage of CdS-NP or PbS-NP is increased, although in different dose ranges. Note, however,
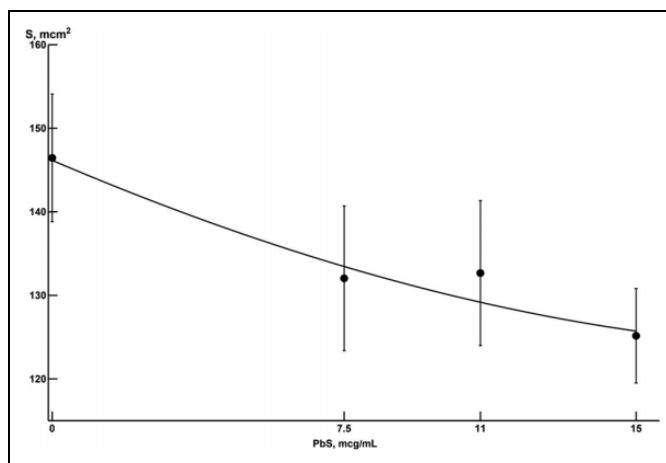
Figure 2. Reduction in ATP-dependent luminescence in endothelium cell culture under exposure to various doses of PbS-NP. The abscissa plots the acting concentrations of nanoparticles in the incubation medium, mg/mL; the ordinate plots the values of the Relative Light Units (RLU) index in the exposed or control groups. The dots indicate the mean values with the standard error of the mean. The equation for the approximating curve is $y = 9.3817 + \frac{1.70016}{1+5.36008e^{0.930363x}} \frac{2.59332}{1+3999.03e^{0.930363x}}$.

Figure 4. Changes in endothelium cell area under exposure to various doses of PbS-NP. The abscissa plots the acting concentrations of nanoparticles in the incubation medium, mcg/mL; the ordinate plots the cell area in mcm^2 in the exposed or control group. The dots indicate the mean values with the standard error of the mean. The equation for the approximating curve is $y = 146.0241.97602 \left(1 - e^{0.12037x}\right) 2.02962x$.
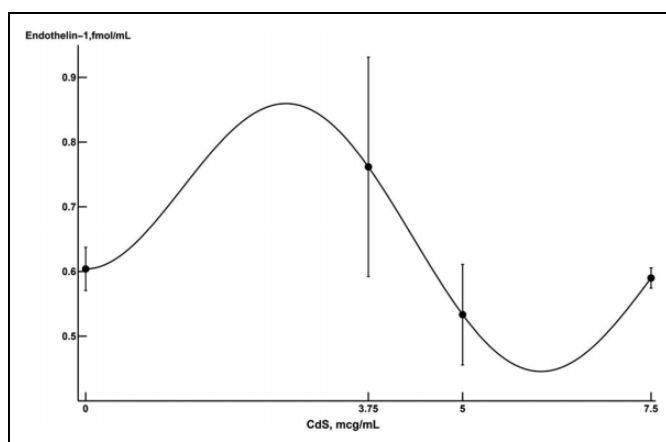

Figure 3. Changes in endothelium cell area under exposure to various doses of CdS-NP. The abscissa plots the acting concentrations of nanoparticles in the incubation medium, mg/mL; the ordinate plots the cell area in mcm^2 in the exposed or control group. The dots indicate the mean values with the standard error of the mean. The equation for the approximating curve is $y = 146.455 + 365.802 J_3(x) \, 767.986 J_4(x) + 453.175 J_5(x)$.

Figure 5. Changes in endothelin-1 concentration in cell lysate under exposure to various doses of CdS-NP. The abscissa plots the acting concentrations of nanoparticles in the incubation medium, mcg/mL; the ordinate plots the endothelin-1 concentration, fmol/mL in the exposed or control group. The dots indicate the mean values with the standard error of the mean. The equation for the approximating curve is $y = 0.604 + 0.83231 \, J_2(x) - 0.657642 \, J_3(x) + 0.333574 \, J_4(x)$.

that in our previous experiment on HL-1 cardiomyocytesС-СЫЛКА for this integral index of cytotoxicity, a similar type of dose-response dependence was observed for PbS-NP only, whereas for CdS-NP it was monotonic and was described well, for instance, by the hyperbolic function.

As seen from Figures 3 and 4, a dependence of the endothelial cell area on the dose of the same factors is, on the contrary, triphasic for CdS-NP and monotonically hyperbolic for PbS-NP.

It is to be emphasized that even when cell size increased somewhat with increasing concentration of CdS-NP in the second phase of the dose-response relationship, it still did not differ significantly from the control value. Statistically highly significant was only the impact of the CdS-NP dose that induced an opposite effect, while this hypotrophic effect was characteristic of all PbS-NP doses tested. Note that in our animal experiments *in vivo* we have obtained a morphometric characteristic which may be matched with endothelial cell hypotrophy under the

impact of the same 2 metals, although in their ionic form rather than as nanoparticle components; the case in point is a considerable thinning of the aortal *tunica intima*.[23]

Taking into consideration results of an animal experiment presented and discussed by Sutunkova et al.,[24] of special interest is the question whether or not the *in vivo* decrease of the serum ET-1 level in rats exposed to NPs under study is due just to reduced ET-1 production by endothelial cells as estimated *in vitro*. As follows from Figures 5 and 6, the answer to this question is affirmative only for the middle range of the CdS-NP doses used while negative for PbS-NP.

As can be seen from the isobolograms constructed on the basis of the RSM model (Figure 7), the combined toxicity of

Figure 6. Changes in endothelin-1 concentration in cell lysate under exposure to various doses of PbS-NP. The abscissa plots the acting concentrations of nanoparticles in the incubation medium, mcg/mL; the ordinate plots the endothelin concentration, fmol/mL in the exposed or control group. The dots indicate the mean values with the standard error of the mean. The equation for the approximating curve is $y = 23.595\left(1 - e^{-0.0399x}\right) - 0.644x + 0.574$.

CdS-NP and PbS-NP is unidirectional and, at the same time, additive for ATP-dependent luminescence reduction but contra-directional for the other 2 effects considered above. Such variability of combined binary action typology, depending, among other things, on which effect it is estimated by, pertains to the important postulates of the theory developed by us as was emphasized in the Introduction. In addition to the ample support for this postulate obtained in *in vivo* experiments, also mentioned in the Introduction, toxicity assessment data obtained *in vitro* for various nanoparticles had demonstrated the same for the combination of NiO-NP and Mn_3O_4-NP on several cell lines[6] and for the combination of CdS-NP and PbS-NP on an HL-1 cardiomyocyte line.[9]

At the same time, these studies (Minigalieva et al., 2017[6] in particular) showed that a specific combined toxicity type characteristic of a particulate's effect on a cell culture and of a seemingly toxicodynamically-related index may mismatch a similar index on organ/system level. The same may be noted when comparing the result shown in Figure 7 with the effect of combined lead-cadmium subchronic intoxication on ET-1 level in blood serum[24]).

These inconsistencies are hardly surprising considering the fact that the outcome of an effect of just 2 toxic agents on any index characterizing the status of a whole animal organism is mediated by a considerably greater number of primary and feedback mechanisms than the outcome of an impact on a cell pulled out from this mechanistic framework (not to mention micro-environmental differences).

Therefore, as early as when discussing the comparative results of assessing the NiO-NP + Mn_3O_4-NP combination on several cell cultures and on rats[6] we emphasized that we consider *in vitro* testing of little practical value for clinically meaningful prediction of types of combined action on the organism. We see no reason so far to give up our point of view.
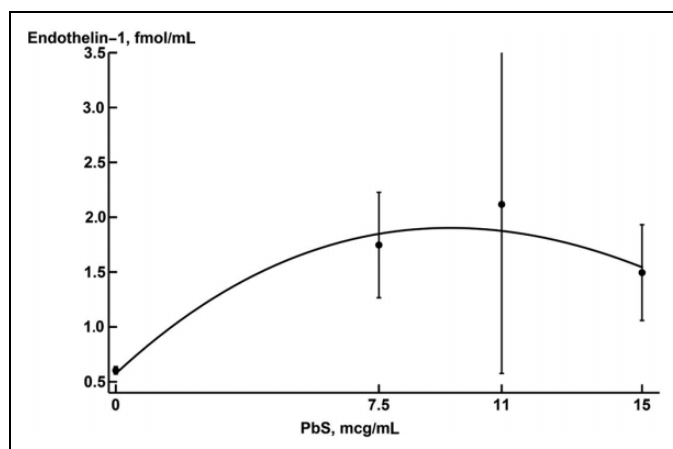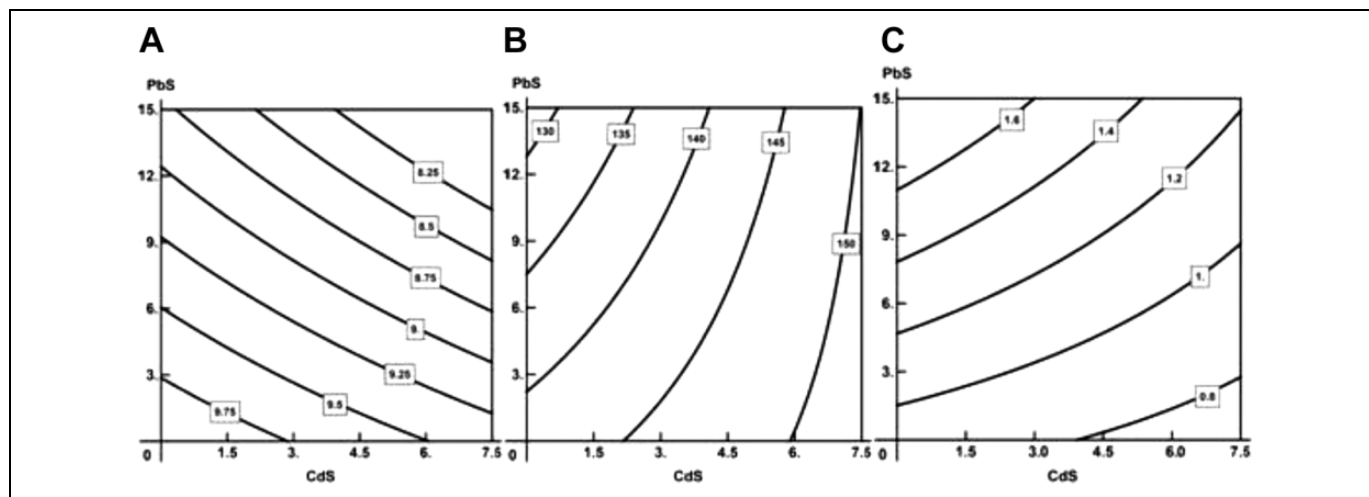
Figure 7. Isobolograms characterizing the combined toxic action of CdS-NP and PbS-NP on an endothelial cell culture as estimated by its effects on (A) ATP-dependent luminescence, (B) cell area and (C) endothelin-1 concentration. Numbers at the axes are respective NP concentrations in mcg/mL; numbers at the isoboles are the values of the effect to which they correspond.

Conclusions

1. It may be assumed that the described by us separately adverse vascular-regulatory effects of animal intoxications with lead and cadmium are mediated by various toxicodynamic mechanisms operating at system and organism levels rather than being connected with the direct cytotoxic action of lead- and cadmium-containing nanoparticles on cells controlling the production of endothelin-1. This assumption is suggested by the fact that in the present experiments in which an established line of arterial endothelial cells was subjected to such direct action, the inhibition of ET-1 production was observed in a certain range of doses for cadmium-containing NPs only, and not for lead-containing ones at all. At the same time, the dose-dependent adverse effect on the viability and size of these cells was more definite for the action of the lead-containing nanoparticles.

2. It has been confirmed once more that the combined binary action typology is essentially variable depending on which effect it is estimated by. However, a specific combined toxicity type characteristic of a particular effect on a cell culture and of a related index of *in vivo* toxicity on organ/system levels may mismatch.

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