Toxicological characterization of bio-active drugs on basis of Iron Fe, Co, and Copper Cu nanopowders

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Abstract. The article presents investigations of toxicological parameters (acute and chronic toxicity, cumulative coefficient) of iron, cobalt, copper and copper oxide nanoparticles with white rats in labs. We have estimated the optimal concentrations of the above mentioned substances with rabbits. We have also studied morphological, physiological and biochemical parameters of the animals when adding the optimal doses to the diet for a long term.

1. Introduction
The authors have studied the biological and catalyst properties of nanomaterials and developing bio-drugs on the basis of metal nanoparticles to be used in livestock-raising and veterinary at the Centre of nanomaterials and nanotechnologies for AIC in Ryazan Agrotechnological University Named after P.A. Kostychev. Investigations have shown that one can use ultra-fine iron, cobalt and copper as non-expensive, nontoxic and highly efficient biological catalysts of biochemical processes in the organism improving physiological state, morphological and biochemical blood parameters increasing activity of the experiment animals’ ferment systems and increasing their productivity and biological value of meat [1,2,3,4].

The bio-drugs study was carried out in different regions of Russia (Moscow, Tula and other oblasts, Krasnodar, Stavropol Territories). We have been studying ultra-fine powders at Ryazan Agrotechnological University Named after P.A. Kostychev since 1997. We have studied the influence of nano-crystal metals on the physiological state of lab and agricultural animals, their productivity and products safety in a case of adding metals to the diet.

For effective use of nano-bio-drugs it is necessary to characterize them toxicologically and determine toxic and optimal concentrations of metals nano-particles for lab animals [5,6].

2. Methods
The aim of the investigations has been determining toxic and optimal doses of nano-bio-drugs on lab animals and morphological-physiologic and biochemical characteristics of animals in a case of long-term adding to the diet the toxicologic doses of the drugs being studied.

For the investigations we have chosen lab non-pedigree white rats and rabbits, breed “Soviet chinchilla”.

We conducted our experiments in 2006-2009 and they included lab and farm tests. [4]
In first experiments we have studied:
1. Metals nano-powders toxicity (acute and chronic toxicity, LD₅₀, cumulative properties).
2. Optimal doses of nano-crystal metals being added to the diet.
3. The effect of nano-crystal metals on the physiologic state, blood morphological-biochemical parameters, health and reproduction of rabbits in the lab.

We have chosen the experiment animals into groups according to the principle of balanced groups-analogues taking into account their sex, age, breed, live weight. The animals have been in the same food and housing conditions. We have taken blood from the side ear vein four- timely at interval of 10 days before morning feeding. We have determined all blood parameters (hemoglobin, erythrocytes, leucocytes, leucocytic formula and blood sedimentation rate) according to unitized methods of clinical diagnostics [7]. We have analyzed the biochemical parameters of blood with spectrophotometer “Spectrum” firm “Abbot” according to unitized methods of clinical lab investigations [8].

We have determined the number of alanine and aspartate transaminase by Wrightman-Frenkel’s colorimetric method; the number of lactate dehydrogenase by the reaction with 2,4-dinitrophenyl diaimide (method of Sevel & Tovarec); the number of alkaline phosphatase by the reaction with p-nitrophenyl phosphate (method of Bessey, Lorie & Brock); the amount of uric acid by the reaction with phosphatotungstic reagent and the number of cholinesterase by acetylcholine chloride hydrolysis with the help of the colorimetric method.

For true development and safe implementation of biologically active drugs based on metals nano-powders in livestock-raising we have developed their vet and toxicological characteristic.

On the first stage of investigations we have determined acute, chronic toxicity and cumulative properties of iron, cobalt and copper nano-powders. We have conducted our investigations in determining acute toxicity in lab conditions. The objects of the investigation have been non-pedigree male rats weighing 180-210 g.

We have got 8 experiment groups 10 animals each and have chosen the doses of each metal being studied taking into account some peculiarities (table 2).

**Table 1.** Feeding diet of lab rats during experiment (in grams per 1 animal a day)

| Fodder and Additives                      | Amount, g |
|------------------------------------------|-----------|
| Grain mixture (oats, millet, wheat, sunflower) | 15        |
| Wheat-bread                               | 4         |
| Grits                                     | 3         |
| Mixed fodder                              | 10        |
| Milk                                      | 8         |
| 2nd category meat                         | 5         |
| Succulent fodder (carrot, beetroot, cabbage) | 10        |
| Greenery                                  | 10        |
| Fish oil                                  | 0.1       |
| Feed yeast                                | 0.2       |
| Bone flour                                | 0.2       |
| Salt                                      | 0.2       |

We have got 8 experiment groups 10 animals each and have chosen the doses of each metal being studied taking into account some peculiarities (table 2).

**Table 2.** Doses of studied drugs for experiment groups

| Groups | Iron Nano-Powder | Copper Nano-Powder | Copper Oxide Nano-Powder | Cobalt Nano-Powder |
|--------|-----------------|--------------------|--------------------------|-------------------|
| Control | -               | -                  | -                        | -                 |
| Experiment 1 | 100 mg/kg | 10 mg/kg | 10 mg/kg | 10 mg/kg |
| Experiment 2 | 500 mg/kg | 50 mg/kg | 20 mg/kg | 50 mg/kg |
| Experiment 3 | 1000 mg/kg | 100 mg/kg | 50 mg/kg | 100 mg/kg |
| Experiment 4 | 1500 mg/kg | 150 mg/kg | 80 mg/kg | 150 mg/kg |
| Experiment 5 | 2000 mg/kg | 200 mg/kg | 100 mg/kg | 200 mg/kg |
| Experiment 6 | 2500 mg/kg | 250 mg/kg | 250 mg/kg | 250 mg/kg |
| Experiment 7 | 3000 mg/kg of live | 300 mg/kg of live | 300 mg/kg of live | 300 mg/kg of live |
We have estimated the average mortal doses for each metal nano-powder a posteriori:

| Metal          | LD50 range      |
|----------------|-----------------|
| Iron           | LD50 - 2130 + 80 mg/kg |
| Copper         | LD50 - 380 + 30 mg/kg |
| Copper oxide   | LD50 - 300 + 80 mg/kg |
| Cobalt         | LD50 - 145 + 10 mg/kg |

The groups getting the maximum concentration of the drug being studied have demonstrated considerable increase of leucocytes in animals’ blood (up to 30 % higher than the control). It is connected with rats’ protective reaction to getting the drug because the leucocytes level as immune factors shows the immune response toxic substances. One can also see considerable increase of bilirubin and its fractions during the experiment (up to 30 %) that is connected with liver cells damage because of the toxic effect of maximum doses of the drug being studied. The increase of crude protein (up to 24 %) and protein metabolism products (uric acid, creatinine) proves the possible damage of the excretory system and kidneys in particular. The experiment animals’ autopsy has proved the biochemical results of the experiment. We have seen liver hyperemia and asphyxic heart.

We have had the experiment in determining sub chronic toxicity and cumulative properties in 3 series during 40 days. On first 5 days every rat has got 1/10 from the existing single dose LD50. Then we have increased the dose by 1.5 times every 5 days. During the experiment we have registered cases of toxicosis and animals’ death and prosected the dead animals.

Based on the experiments we have estimated the chronic median lethal dose:
- for iron nano-powder: chronic LD50 = 25 743.8 mg/kg of live weight;
- for copper nano-powder: chronic LD50 = 4 120.5 mg/kg of live weight;
- for copper oxide nano-powder: chronic LD50 = 1 543.8 mg/kg of live weight;
- for cobalt nano-powder: chronic LD50 = 1 780.0 mg/kg of live weight.

The cumulative coefficient (K_cum) for nano-powders has been as follows:
- iron – 12.09; copper – 10.84; copper oxide – 5.15; cobalt – 12.27.

According to the standard classification some cumulative coefficients prove that the accumulation of all the metals being studied has been mild.

Taking into account the results we have got one can say that nano-powders of iron, cobalt, copper and copper oxide do not posses any cumulative properties and according to GOST 12.1.007-76 “Hazardous substances” can be attributed to the 4th class of hazard, i.e. inconsiderably hazardous substances.

3. Results and Discussion

To determine the metabolism state of the experiment animals in a case of feeding them with copper nano-powder we have estimated the dynamics of some blood biochemical parameters characterizing this or that metabolic process (table 3).

We have carried out our investigations with non-pedigree male rats in the lab. According to the principle of animals-analogues we have chosen 20 rats. We have taken the experiment animals’ blood sampling and autopsy in the lab together with some specialists.

The experiment groups animals have got an increase of crude protein and the level of residual nitrogen during the whole experiment as compared with initial rates on the average by 5.4-9.1 % and 9.2 – 20.6 % (P<0.05) correspondingly.

The blood of the experiment rats has not practically differed by morphological parameters as compared with the control groups. The average results of erythrocytes, hemoglobin, leucocytes, color index and leukogram have been according to the norm. The study of main biochemical parameters has not educed any negative effects of the nano-powders usage. On the contrary there has been some positive shift in the dynamics of glucose concentration and the level of cholesterol and urea total...
bilirubin and creatinine has declined. Therefore, the metabolic state of the experiment animals when feeding them with nano-powders has improved.

### Table 3. The Experiment animals’ blood biochemical parameters

| Group | Time of Investigation, days | Crude Protein, g/l | Glucose, mol/l | Total Bilirubin, mcmol/l | Urea, mol/l | Creatinine, mcml/l |
|-------|-----------------------------|--------------------|----------------|-------------------------|-------------|------------------|
|       | Initial | 10 | 20 | 30 | 60 | Initial | 10 | 20 | 30 | 60 |
| 1st (1/40 of LD<sub>50</sub>) | 67.0±0.5 | 68.7±0.05 | 68.5±0.03 | 70.5±0.05 | 71.1±0.04 | 3.12±0.07 | 3.18±0.001 | 3.18±0.001 | 3.10±0.001 |
| 2nd (1/10 of LD<sub>50</sub>) | 65.7±0.02 | 66.6±0.03 | 69.4±0.07 | 71.7±0.07 | 72.1±0.06 | 2.83±0.002 | 2.87±0.007 | 2.87±0.007 | 2.85±0.003 |
| 3d (1/4 of LD<sub>50</sub>) | 66.7±0.09 | 67.6±0.03 | 70.2±0.03 | 72.0±0.09 | 71.1±0.08 | 2.83±0.002 | 2.87±0.007 | 2.87±0.007 | 2.85±0.003 |
| Control | 67.0±0.6 | 66.7±0.05 | 99.7±0.06 | 65.4±0.05 | 67.0±0.7 | 3.12±0.07 | 3.18±0.001 | 3.18±0.001 | 3.10±0.001 |

Residual Nitrogen, mg %

| Group | Time of Investigation, days | Crude Protein, g/l | Glucose, mol/l | Total Bilirubin, mcmol/l | Urea, mol/l | Creatinine, mcml/l |
|-------|-----------------------------|--------------------|----------------|-------------------------|-------------|------------------|
|       | Initial | 10 | 20 | 30 | 60 | Initial | 10 | 20 | 30 | 60 |
| 1st (1/40 of LD<sub>50</sub>) | 33.3±0.04 | 34.5±0.07 | 36.6±0.06 | 39.2±0.04 | 38.4±0.03 | 3.12±0.07 | 3.18±0.001 | 3.18±0.001 | 3.10±0.001 |
| 2nd (1/10 of LD<sub>50</sub>) | 36.2±0.02 | 34.8±0.04 | 37.6±0.04 | 39.5±0.04 | 38.3±0.04 | 2.83±0.002 | 2.87±0.007 | 2.87±0.007 | 2.85±0.003 |
| 3d (1/4 of LD<sub>50</sub>) | 32.6±0.08 | 36.2±0.09 | 38.3±0.03 | 39.3±0.06 | 39.0±0.3 | 2.83±0.002 | 2.87±0.007 | 2.87±0.007 | 2.85±0.003 |
| Control | 33.0±0.3 | 35.5±0.07 | 35.7±0.03 | 35.0±0.2 | 36.1±0.04 | 3.12±0.07 | 3.18±0.001 | 3.18±0.001 | 3.10±0.001 |

Note: - P ≤ 0.05

We have determined the optimal doses of metals nano-powders when adding them to the diet of the lab animals with rabbits breed “Soviet chinchilla” aged 30 days. We have got 10 experiment groups each having 6 rabbits.
To add metals nano-powders to the feeding diet we have used the mixed fodder treated with the drugs suspension. Every 10 days of the experiment and 10 days after it we have weighed the experiment animals and had morphological and biochemical tests of the experiment animals’ blood and supervised the animals’ general development, appetite and activity. The rabbits have been kept in conditions recommended for their management and breeding in a case of twice-a-day feeding. The influence of different doses of ultra-fine metals on the live weight of the control and experiment animals is presented in table 5.

One can consider 0.08 mg/kg of the live weight to be the optimal dose of the iron nano-powder. This concentration has promoted the increase of the animals’ weight up to 7.5 % in 30 days after giving the drug. The rest concentrations have had less effect: the dose of 0.16 mg/kg of the live weight has given 5.5 % weight increase and the dose of 0.24 mg/kg has brought a 3.9 % increase as compared with the control.

The optimal dose for cobalt nano-powder has been 0.02 mgr/kg of the live weight that let increase the live weight by 4.8 % higher than the control. For copper nanopowder the optimal dose has been 0.04 mg/kg of the live weight a day that has increased the rabbits live weight by 3.8 as compared with the control. The increase of copper nanoowder dose up to 0.12 mg/kg of the live weight has negatively influenced the animals’ weight gain that has been 0.8 % lower than that in the control group in 30 days of the experiment. Moreover, the animals in this group have been less active in comparison with the control ones. Therefore, high concentration copper has oppressed the rabbits’ health.

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**Table 4.** The rabbits’ feeding diet when using the combined feeding type (g per 1 animal)

| Fodder                        | Age (days) | Replacement older than 120 days |
|-------------------------------|------------|---------------------------------|
|                               | 30-60      | 61-120                          | Live weight (kg) |
| Grain (oats), g               | 60         | 80                              | 80              |
| Mixed fodder, g               | 10         | 20                              | 30              |
| Sunflower coarse meal, g      | 5          | 15                              | 10              |
| Succulent foders, g (carrot, beetroot, cabbage, potato) | 50         | 120                             | 150             |
| Meadow hay, g                 | 80         | 100                             | 150             |
| Meadow grass, g               | 250        | 400                             | 450             |
| Fish oil, g                   | 0.3        | 0.5                             | 0.5             |
| Bone flour, g                 | 2.0        | 2.0                             | 2.0             |
| Feed yeast, g                 | 0.2        | 0.4                             | 0.4             |
| Salt, g                       | 0.6        | 1.0                             | 1.2             |
| Fodder units (EFU)            | 0.11-0.15  | 0.16-0.20                       | 0.21-0.23       |
| Metabolic energy, mJ          | 1.10-1.45  | 1.61-2.00                       | 2.09-2.30       |
| Dry matter, g                 | 93-130     | 130-195                         | 200-220         |
| Crude protein, g              | 18-27      | 27-37                           | 34-37           |
| Digestible protein, g         | 14-21      | 21-28                           | 26-29           |
| Crude fiber, g                | 17-23      | 20-32                           | 35-39           |
| Calcium, g                    | 0.8-1.0    | 1.1-1.4                         | 1.5-1.7         |
| Phosphorus, g                 | 0.6-0.7    | 0.7-0.9                         | 0.9-1.1         |
| Carotene, mg                  | 0.8-1.4    | 1.5-2.0                         | 2.4-2.6         |
| Vitamin D, mg                 | 100-170    | 170-240                         | 320-380         |
| Vitamin E, mg                 | 2.0-3.4    | 3.4-4.8                         | 6.4-7.6         |

**Table 5.** Live weight of the lab rabbits, g
The cancellation of copper NP has not led to the weight decline of the experiment animals. On the contrary the tendency of increasing the body weight has remained even 10 days after the cancellation of metals ultra-fine powders (table 5).

During the investigations all morphological parameters of the experiment rabbits’ blood have been within the physiological norm.

3.1. Metals nano-powders influence on physiological parameters of rabbits and blood morphological biochemical indexes.

For the experiment we have formed the following groups 8 animals each: group 1 – control; group 2 – experiment – 0.08 mg/kg iron ultra-fine powder; group 3 – experiment – 0.02 mg/kg cobalt ultra-fine powder; group 4 – experiment – 0.04 mg/kg copper ultra-fine powder a day. The experiment has lasted for 60 days. Every 10 days we have weighed the control and experiment animals. The age of the animals at the beginning of the experiment has been 30 days. The animals in all 4 groups have steadily gained the weight, been active and had good appetite. We have seen some positive results of nano-crystal metals usage in 10 days of the experiment. Adding iron nanopowder to the diet has increased the rabbits’ live weight by 8.8 % in 30 days and by the end of the experiment by 11.7 % higher than the control. Cobalt nano-powder has also promoted the rabbits’ live weight increase by 5.6 % in 30 days and by 7.8% in 60 days. The increase in a case with nano-copper has been 6.3 % higher than the control at the end of the experiment.

We have carried out a simultaneous investigation of morphological and biochemical parameters of the experiment animals’ blood. We have chosen 4 animals from each experiment group. Blood sampling has taken place every 10 days before morning feeding. Before the experiment all blood parameters have been within the physiological norm. Table 6 presents the results of investigating the blood of the control and experiment animals in 60 days of the experiment.

| Groups | Before Experiment | In 10 Days | In 20 Days | In 30 Days | 10 Days after Experiment |
|--------|------------------|------------|------------|------------|------------------------|
| Control | 845              | 1010       | 1145       | 1260       | 1510                   |
| Group 1 Fe NP 0.08 mg/kg | 840 | 1130 | 1230 | 1355 | 1660 |
| Group 2 Fe NP 0.16 mg/kg | 830 | 1110 | 1180 | 1330 | 1625 |
| Group 3 Fe NP 0.24 mg/kg | 840 | 1090 | 1165 | 1310 | 1610 |
| Group 4 Co NP 0.01 mg/kg | 850 | 1120 | 1195 | 1290 | 1620 |
| Group 5 Co NP 0.02 mg/kg | 840 | 1125 | 1210 | 1320 | 1630 |
| Group 6 Co NP 0.03 mg/kg | 835 | 1100 | 1170 | 1275 | 1630 |
| Group 7 Cu NP 0.04 mg/kg | 830 | 1090 | 1185 | 1305 | 1590 |
| Group 8 Cu NP 0.08 mg/kg | 840 | 1070 | 1160 | 1275 | 1560 |
| Group 9 Cu NP 0.12 mg/kg | 845 | 1045 | 1150 | 1250 | 1505 |

Note: - P ≤ 0.05
The rabbits that have got ultra-fine metals as an additive have shown the true increase of erythrocytes and hemoglobin.

The iron nano-powder has increased erythrocytes by 9.3 % and hemoglobin by 9.1 % in 60 days of the experiment as compared with the control that has positively influenced the animals' physiology. By the end of the experiment there has been a 29.3 % increase of leucocytes and the leukogram change, i.e. the increase of lymphocytes (by 8 %) and the decline of granulocytes by 8 %. There has also been an increase of total protein in serum (by 10.5 %) and α1 - globulins (by 2.5 %) that has caused the decline of α2 - globulins and albumins. The rest parameters have had inconsiderable deviations from the control.

One can see similar changes in other experiment groups as well. Cobalt nano-powder has increased erythrocytes by 5.5 %, hemoglobin by 10 %, leucocytes by 9.8 %, lymphocytes by 10 % and total protein by 16.2 %.

The copper nano-powder has increased the level of erythrocytes and hemoglobin (by 7.4 % and 7.3 %), leucocytes and lymphocytes (by 24.4 % and 7 %). All serum parameters in a case of nano-crystal metals have been within the physiological norm. But there has been an increase of total protein at the expense of the globulin fraction and α1 - and β - globulins in particular. It is known that α1 - and β - globulins synthesize in the liver and serve active transmitting agents of different blood substances. The increase of these fractions leads to strengthening the active transfer of carbohydrates and lipids to tissues that influences the number and activity of globulins. This in its turn activates metabolism. γ - globulins have had the same character of changes. At the expense of γ -globulins increase as compared with the control there has been an increase of the immune-biological reactivity as they are protective anti-substances (immune globulins) and are responsible for the specific immune response. These parameters have changed most vividly in a case of adding Iron and cobalt nanopowders.

The immune system that is structurally and functionally organized combination of lymphoid cells interacting with each other and some secondary cell elements on different stages of immune genesis can be an indicator reflecting nanomaterials effect on the organism. If one takes into account that ultra-fine microelements activate immune, ferment and humoral systems of the organism increasing metabolism and better digestion of nutrients then we can use them as bio-additives.

Female and male rabbits after getting the offspring have been dissected to see the state of their internal organs. At that we have discovered the total absence of internal organs pathologies: the liver, the kidneys, the heart, the lungs, the bowels have been in norm, without any external changes, had normal size. The structure of each organ has been in agreement with the norm.
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