Toxicological safety control and dosage parameters of the pharmacological agent SPAO for chickens

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Abstract. To reduce economic losses caused by technological factors affecting chickens, a pharmacological complex SPAO (a stress protector antioxidant) was developed. SPAO is a pharmacological composition containing lithium citrate, vitamins, and other substances that affect the metabolism of chickens. The toxicological studies proved that the SPAO belongs to the group of low-toxic substances that do not have locally irritating properties. With a single oral administration at a dose of 6773.33 mg per 1 kg of body weight, there were no toxic effects. It is a low-hazard substance (relatively harmless). As a result of assessment of the dose-effect relationship, when the level of corticosterone in the blood of chickens was used as a marker sign of activation of the hypothalamic-pituitary-adrenal system, the lowest dose providing a pronounced anti-stress pharmacological effect is 185 mg/kg according to the scheme (two days and a day before exposure to stress factors). Long-term administration of the SPAO-complex at a dose of 185–1850 mg per 1 kg of body weight does not cause the death of experimental animals. In therapeutic doses, the SPAO-complex does not adversely affect the general condition of experimental animals, digestion and urination, the central nervous system and the morphology of internal organs. At doses exceeding therapeutic ones by 5–10 times, within 40 days of use, it increases the heart weight, causes dystrophic phenomena in liver and increases the volume of spleen, without a significant effect on the weight of this organ.

1. Introduction
Currently, there is irrefutable evidence of the leading role of stresses in the pathological processes in the body of chickens and a decrease in the economic efficiency of industrial poultry farming due to stresses. It is not possible to get rid of stresses in the conditions of industrial poultry farming as the body reacts to any technological impact by developing adaptive reactions, including stress. In this regard, special attention should be paid to research in the field of development of effective complex-acting drugs that help minimize negative effects of stresses [1].

Stress prevention is based on the use of drugs of different pharmacological groups. Studies have shown that the most effective approach is the use of pharmacological agents with a specific anti-stress effect in combination with substances that have metabolic effect on the body and contribute to synergies from their combined use [2]. This aspect requires the development and industrial production of new highly effective and safe drugs for the prevention of stresses in chickens. One of the directions may be the study and development of effective recipes for pharmacological compositions consisting of pharmaceutical substances based on the synergism of individual substances and providing a comprehensive therapeutic effect.
To reduce economic losses caused by stresses, SPAO, a stress protector antioxidant, has been developed. SPAO is a pharmacological composition containing lithium citrate, vitamins, and other substances that influence the metabolism of chickens. SPAO is a white powder that is highly soluble in water (16.95 g/100 g at 20 °C) [3].

The purpose of this work is to study toxic and local irritant properties of SPAO.

2. Materials and methods
The experiments were performed in accordance with recommendations by Erik Walum (1998). The toxicological properties of SPAO were studied by determining parameters of acute and subchronic toxicity, as well as local irritating effects. Experiments were carried out on white mice, rats, rabbits and guinea pigs.

Acute toxicity was determined with a single administration at maximum doses for each of the methods. Acute toxicity was determined on 20 white mice. The animals were divided into 2 groups consisting of 10 mice weighing 20–22 g. The animals of the experimental group were given a solution inside using a probe. The SPAO complex was used at the maximum dose, taking into account the solubility of 6773.33 mg/kg, the second group of mice was control, they were injected with a similar amount of distilled water.

The toxicity experiment was conducted on 10 chinchilla rabbits and 10 guinea pigs. Their back hair was cut to the right and left of the spine. The right side served for the application of the drug, the left side – for the control (distilled water). The thickness of the skin fold was determined using a caliper.

When examining the effect of SPAO on the mucous membranes, 2 drops of a saturated solution of the SPAO-complex were applied into the conjunctival sac of the right eye, the same amount of distilled water was dropped into the left eye for control. After making the drug, the nasolacrimal canal of the eye was pressed for 1 minute. Observations were carried out within 3 days.

Due to the low acute toxicity of the SPAO complex, the determination of LD50 becomes impracticable. Accordingly, the determination of the dose range for the study of subchronic toxicity was calculated for the therapeutic dose. The effective therapeutic dose was determined by assessing the effect of the SPAO-complex on the level of corticosterone in the blood of chickens in response to the acute stressing factor. An intracutaneous injection of 70 % turpentine solution into the area of the scallop of a chicken at a dose of 0.1 ml was used as an irritant. Intradermal administration of the turpentine solution causes adaptive reactions and stress. The method is used to model stress in chickens at the anxiety stage or stress orientation stage [3]. To assess the severity of the adaptive process in chickens, glucocorticoid hormones in the blood were determined; this method is the most common for diagnosing the development of stress in birds [4]. According to some authors, the highest level of corticosterone is observed 30 minutes after the stressful effect [5]. The concentration of corticosterone in the blood serum was studied using an enzyme-linked immunosorbent assay (ELISA) on a Tecan Sunrise analyzer at a wavelength of 450 nm; ELISA kits “DRG Corticosterone ELISA KIT” were used for determination. The smallest measurable corticosterone concentration is less than 1.631 nmol/L. Cross-reaction to corticosterone 100 %, progesterone 7.4 %, deoxycorticosterone 3.4 %, 11-deoxycorticosterone 1.6 %, cortisol 0.3 %, other steroids was less than 0.1 % [6].

Blood sampling was carried out 30 minutes after intradermal injection of 70 % turpentine oil solution. For the experimental groups, two and three days before the stressful effect and on the day of the stressful effect, the SPAO at doses of 150, 185, 220 and 250 mg/kg was applied according to the scheme two days and a day before exposure to the stressful factor in the form of intradermal administration 70 % turpentine solution. In each group, corticosterone levels were measured in 10 chickens. The dose range of 150–250 mg/kg of body weight was obtained experimentally in preliminary screening studies.

Subchronic toxicity was evaluated in 24 non-linear rats weighing 244.4 ± 26.0 g, which were divided into 4 groups consisting of 6 animals. The SPAO complex was used with water by individual evaporation. The first experimental group received the SPAO-complex at a therapeutic dose of 185 mg/kg. The second experimental group received SPAO at a five-fold therapeutic dose –
925 mg/kg body weight, the third group – at a ten-fold therapeutic dose – 1850 mg/kg. The fourth group served as a control one and received saline at a similar dose.

The SPAO complex was applied for 40 days. During the experiment, changes in the general condition and behavior of rats, body weight, mobility, appetite, and coat and skin were observed. After the end of the chronic experiment, blood was taken decapitation under the influence of diethyl ether. Rat autopsies were performed immediately after euthanasia.

The statistical analysis of the experimental data was carried out using the STATISTICA 12 program. Comparative data are average with the standard deviation. To assess intergroup differences, the nonparametric Mann-Whitney U-test was used; to assess the level of statistical differences within the group at different chronological intervals, the Wilcoxon test was used. The level of statistical significance was taken equal to 0.05.

3. Results and Discussion

It was found that the SPAO-complex when administered at the maximum possible dose did not cause the death of experimental mice. No changes in behavior were observed; reflexes were preserved.

Body weight gain (Table 1) was proportional to the age. Statistical differences in body weight changes were not observed.

Table 1. Dynamics of changes in body weight of mice treated with the maximum SPAO dose, g

| Indicator                | Experiment       | Group          |
|-------------------------|------------------|----------------|
| Before treating         | 23.00±2.00       | 23.20±1.79     |
| Body weight on the 5th day | 23.30±1.68       | 23.40±1.98     |
| Body weight on the 14th day | 23.60±1.34       | 23.80±2.59     |
| Weight gain over the observation period | 0.60 | 0.60 |

Therefore, according to the degree of exposure to warm-blooded animals, the SPAO-complex belongs to low-hazard substances (Relatively harmless according to the classification of Loomis & Hayes, 1996 [7, 8]).

Evaluation of the local irritating properties when applied to the skin and mucous membranes revealed no changes in the place of application of SPAO both during its use and the observation period. The elasticity of the skin of experimental animals preserves. When palpating the areas of application, an increased pain reaction was not observed. Redness, swelling of the skin and hemorrhage were not identified.

When examining the action of SPAO on the mucous membranes, 2 drops were applied into the conjunctival sac of the right eye, the same amount of distilled water was dropped in the left eye for control. As a result of observations of conjunctival hyperemia and signs of injection of blood vessels of the eye, clouding of the cornea and narrowing of the pupil were not observed.

Based on the detected signs, the locally irritating reaction when using saturated SPAO was negative.

Table 2 presents the results of determining the concentration of corticosterone depending on the dose of SPAO.

A statistical analysis of the differences between the experimental and control groups indicates pronounced statistical differences in the level of corticosterone observed when using the drug at doses of 185, 220 and 250 mg/kg. The results indicate that the use of SPAO at a dose of 150 mg/kg cannot provide an anti-stress pharmacological effect when applied according to the scheme two days and a day before exposure to a stressing factor. The lowest dose range, providing a pronounced anti-stress
pharmacological effect, is 185–250 mg/kg according to the scheme two days and a day before exposure to the stress factor. Given that the SPAO-complex is planned to be used in conditions of industrial poultry farming based on economic factors and ensuring toxicological safety when using a pharmacological agent, the therapeutic dose can be 185 mg/kg of chicken body weight.

Table 2. Dependence of the concentration of corticosterone on the SPAO dose

| No of the group | SPAO dose c | The concentration of corticosterone in the blood serum of chickens, nmol/l | Statistical significance of differences between the experimental and control groups |
|----------------|-------------|--------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| 1              | 150 mg/kg   | 36.20±8.066                                                                     | P=0.1297                                                                         |
| 2              | 185 mg/kg   | 30.00±7.468                                                                     | P=0.0201                                                                         |
| 3              | 220 mg/kg   | 28.70±7.718                                                                     | P=0.0134                                                                         |
| 4              | 250 mg/kg   | 30.20±8.390                                                                     | P=0.0235                                                                         |
| Control        | SPAO was not used | 46.70±19.27                                                                      | P=1.0000                                                                         |

When studying the subchronic toxicity, the SPAO-complex did not cause the death of rats during the 40 day observation. During the observation, no deviations in behavior were found, and external signs of the neurotoxic action of the SPAO complex were not observed.

Data on the long-term effects of the SPAO-complex on the body weight of experimental rats are presented in Table 3.

Table 3. The influence of SPAO on the body weight of laboratory rats, g

| Indicator                | 1       | 2       | 3       | 4       |
|--------------------------|---------|---------|---------|---------|
| Before treating          | 244.0±22.1 | 245.0±24.2 | 253.3±32.9 | 245.0±20.7 |
| Body weight on the seventh day | 242.1±24.6 | 243.3±22.2 | 242.8±31.4 | 243.3±19.4 |
| Body weight on the 14th day | 246.4±23.4 | 238.6±25.1 | 242.0±33.5 | 245.3±18.5 |
| Body weight on the 21st day | 250.2±20.1 | 238.6±25.5 | 245.5±31.8 | 250.0±18.9 |
| Body weight on the 28th day | 253.2±22.7 | 238.6±26.8 | 243.3±33.7 | 254.6±17.8 |
| Body weight on the 35th day | 254.1±24.1 | 244.8±25.4 | 248.3±33.7 | 257.5±21.2 |
| Body weight on the 40th day | 254.2±28.6 | 252.8±22.6 | 253.5±30.2 | 261.0±23.9 |
| Body weight gain          | 10.2    | 7.8     | 0.2     | 16.0    |

The data presented in Table 2 indicate the absence of a pronounced toxic effect of the SPAO-complex at the studied doses. However, there is a statistically unexpressed relationship between a decrease in body weight gain and the applied dose of SPAO. Thus, in the control group, an increase in body weight was observed at the level of 6.1 % of the initial weight, when applying the studied pharmacological composition at a therapeutic dose; an increase in body weight was 4.0 % of the initial body weight when used at a five-fold dose; and by 3.1 % when using a ten-fold dose; at the end of the observation period the body weight corresponds to the initial body weight.

According to the pathological study of the control group, the internal organs are anatomically correctly located. The serous membranes of the cavities are smooth and shiny. The lungs are gray-pink, of normal size and shape, symmetrically located. The pleura is smooth and even. The heart is of normal size, the pericardium and epicardium are smooth, shiny, the myocardium is bright red, dense. The liver corresponds to the size of the body, the edges are sharp, the capsule is smooth; on the surface
and on the section, it is dark cherry, the pattern is pronounced, the gall bladder is filled with liquid yellow-green bile, the patency of the bile ducts is not impaired. The serous membrane of the stomach and intestines is gray, its surface is smooth, even, shiny. The mucous membrane of the stomach, small and large intestines is bright pink, smooth and shiny. The spleen is elongated, with sharp edges and a smooth capsule. The kidneys are symmetrical, dark cherry.

In rats of the first experimental group receiving SPAO at a therapeutic dose, the autopsy pattern corresponds to the above data. Signs of intoxication were not found. In 50.0 % of rats of the second group, autopsy revealed pathological processes in the form of changes in the color of the liver and the size of the spleen. The spleen is slightly increased; its edges are rounded. There is heterogeneity in the color of the liver in the form of areas with darker and lighter colors, the edges are sharp, the organ is not enlarged. In 83.0 % of rats of the fourth experimental group, signs similar to the ones in the third group were observed, accompanied by more extensive foci with uneven coloring on the surface of the liver, the flabby liver was easily torn, the edges were sharp. Similar changes are observed when testing the subchronic toxicity of many low-toxic compounds, for example, an extract of ethyl acetate isolated from bay leaves and other chemical and natural compounds [9, 10].

Table 4. The mass of the internal organs of rats treated with SPAO, %

| Organs   | 1          | 2          | 3          | 4          |
|----------|------------|------------|------------|------------|
| Heart    | 0.332±0.018| 0.360±0.021| 0.377±0.028| 0.327±0.016|
|          | P=0.4590   | P=0.010    | P=0.004    |            |
| Liver    | 3.681±0.306| 3.609±0.328| 3.356±0.236| 3.705±0.311|
|          | P=0.8512   | P=0.6114   | P=0.0534   |            |
| Spleen   | 0.292±0.034| 0.301±0.061| 0.337±0.063| 0.294±0.026|
|          | P=0.8361   | P=0.8081   | P=0.1591   |            |
| Kidneys  | 0.592±0.028| 0.642±0.019| 0.634±0.045| 0.584±0.068|
|          | P=0.7851   | P=0.0719   | P=0.1633   |            |

The prolonged used of high doses of SPAO had a pronounced effect on heart weight. As for the control group, administration of five-fold therapeutic doses led to an increase in heart weight by 9.2 %, and the use of ten-fold doses increased the heart weight by 13.3 %. Therapeutic doses of SPAO used within 40 days did not lead to a statistically significant increase in the heart weight.

The liver mass in the experimental groups decreased. Statistically significant changes were detected only under the influence of ten-fold doses, the indicator was 10.4 % lower. Despite the predominant increase in the size of spleen, its weight is statistically equal to the weight of animals of the control group.

The pathological changes caused by the prolonged use of the SPAO-complex at doses which exceed the therapeutic ones five and ten times are characteristic of poisoning with lithium salts. Considering that the SPAO complex is planned to be used for more than five days, it is safe to use it at therapeutic doses. The long-term use of the SPAO complex at higher doses requires additional research.

4. Conclusion

1. SPAO is a group of low-toxic substances that do not have locally irritating properties. With a single oral administration at a dose of 6773.33 mg per 1 kg of body weight, no signs of toxic effects were found. By the degree of exposure, it can be attributed to low-hazard substances (relatively harmless).

2. As a result of assessment of the dose-effect relationship, when the level of corticosterone in the blood of chickens was used as a marker for the activation of the hypothalamic-pituitary-adrenal system, the lowest dose providing a pronounced anti-stress pharmacological effect is 185 mg/kg according to the two-day schedule and the day before exposure to the stress factor.
3. Long-term administration of the SPAO-complex at a dose range from 185 mg to 1850 mg per 1 kg of body weight does not cause the death of experimental animals. At therapeutic doses, the SPAO-complex does not adversely affect the general condition of experimental animals, digestion and urination, the central nervous system and the morphology of internal organs. At doses exceeding therapeutic ones by 5–10 times within 40 days of use, it causes an increase in heart mass, dystrophic phenomena in the liver and an increase in the volume of the spleen, without a significant effect on the mass of these organs.

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