



## Comparative assessment of various methods of studying the skin toxicity of a wound-healing drug

V. I. Kushnir<sup>1</sup>, I. M. Kushnir<sup>1</sup>, I. P. Patereha<sup>1</sup>, O. T. Kutsan<sup>2</sup>, O. M. Zhovnir<sup>2</sup>, B. V. Gutyj<sup>3</sup>

<sup>1</sup>State Scientific-Research Control Institute of Veterinary Medicinal Products and Feed Additives, Donetska Str., 11, Lviv, 79019, Ukraine

<sup>2</sup>Institute of Veterinary Medicine of the National Academy of Sciences of Ukraine, Donetska str, 30, Kyiv, 03151, Ukraine

<sup>3</sup>Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies, Pekarska Str., 50, Lviv, 79010, Ukraine

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### Correspondence author

Ihor Kushnir

Tel.: +38-096-367-31-37

E-mail: igorku70@gmail.com

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### Abstract

The article presents the results of studying the subacute skin toxicity of a wound-healing cream based on betamethasone dipropionate, gentamicin sulfate, and clotrimazole. According to the Organization for Economic Cooperation and Development (OECD No. 410), the research was conducted using the classic method. When studying the effect of a wound-healing agent on the body of animals during long-term 28-day dermal application by the classical method in animals of the I, II, and III experimental groups, which received the drug in a dose of 0.5, 2.5, and 5 ml/kg body weight; respectively no significant changes were found in the body weight of the animals and the weight coefficients of the liver, heart, spleen, kidneys, and lungs. At the same time, in the animals of the II and III research groups, a slight increase in the concentration of hemoglobin was noted, respectively, by 1.3 and 3.9 %, the level of urea – 5.3 and 11.2 %, the activity of AST – 9.8 and 14.9 % compared to the indicators of animals of the control group. In these groups, there was a decrease in total protein concentration by 5.5 and 6.8 %, creatinine level by 3.1 and 5.3 %, and ALT activity by 6.5 and 9.7 %. The studies conducted according to the OECD No. 410 make it possible to obtain more informative data on the toxic effect of the investigated agent. In particular, it was established that the use of the researched drug in animals of the I, II, and III experimental groups caused a decrease in the weight coefficients of the spleen mass, respectively, by 19.1 (P < 0.05) and 28.3 % (P < 0.05), hearts – 3.6, 4.6 and 7.7 %, kidneys – 4.9, 6.5 and 10.4 % and animal body weight – 4.5, 5.4 and 6.4 %, hemoglobin concentrations – 2.6, 2.9 and 4.4 %, leukocyte counts – 21.4, 16.1 and 15.1 %, hematocrit values – 1.9, 3, 6 and 4.0 %. The average hemoglobin content in the erythrocyte (MSN) also decreased by 8.3, 6.9, and 5.1 %, and the average erythrocyte (MCV) volume was 5.0, 4.7, and 1.7 %. In addition, an increase in weight coefficients of liver mass was established by 10.8 (P < 0.05), 11.8 (P < 0.05), and 15.6 % (P < 0.05). When studying the effect of the researched agent during 28-day dermal application on biochemical indicators of blood serum in animals of the first experimental group, a decrease in the concentration of total protein by 5.8 % was established. Under these conditions, an increase in creatinine level, urea level, ALT, and AST was established, respectively, by 6.2, 18.8 (P < 0.05), 3.8, and 14.7 % (P < 0.05). It should be noted that the use of the researched product in animals of the II and III experimental groups caused an increase in the level of urea, respectively, by 28.6 and 35.7 % (P < 0.05), creatinine by 8.4 and 3.5 %, AST – 16.4 (P < 0.05) and 11.3 % and the activity of ALT – 8.5 and 11.0 %.

**Keywords:** subacute toxicity, laboratory rats, OECD test No. 410, internal organs, hematological indicators, biochemical indicators.

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### 1. Introduction

An essential stage in developing new veterinary drugs is the determination of their safety and effectiveness, which includes conducting a wide range of preclinical studies (Venkatesan & Ramanathan, 2017; Vasylyev et al., 2021; Karpenko et al., 2022; Martyshuk et al., 2022). Special attention is paid to the long-term use of the researched means. Long-term use of drugs provides information on their toxicity, target organs, the dose-response relationship for each toxicity endpoint, reactions to toxic metabolites formed in

the body, delayed reactions, cumulative effects, and information on reversibility or irreversibility of the effect. The obtained research results allow for obtaining data on the possible danger to the health of the macroorganism and serve as the basis for establishing the duration and frequency of practical application of the researched agent in production conditions (Scarpella et al., 2015).

Among the wide range of veterinary medicines, preparations for external use, particularly ointment forms, insect acaricides, etc., deserve special attention. The effectiveness of these drugs depends on the solubility, stability, physical

and chemical properties of the active substance, and the ability to absorb skin (Malkinson, 1960; Stoughton, 1964; Stoughton, 1965; Vinson et al., 1965; Bilous et al., 2014) the concentration of the active substance, the pH of the environment, and the ability of the tested agent to be absorbed on the surface of the skin (Stoughton, 1964; Vinson et al., 1965). Such medicines provide transdermal delivery of active substances and contribute to wound healing and reduced swelling and itching (Bilous et al., 2010).

In connection with the integration of Ukraine into European structures, the established national rules for determining toxicity need to be revised and harmonized with European norms. At the same time, new requirements for conducting toxicological studies are established.

Today, in the countries of the European Union, preclinical studies are conducted following EU Regulations (Council Regulation (EC) No 440/2008) and recommendations of the Organization for Economic Cooperation and Development (OECD). The use of OECD recommendations is based on assessment and can evaluate the substance according to the degree of danger and systematize the results of studies. (OECD, 2017). In addition, it should be noted that these recommendations are a unique tool for assessing the potential impact of chemicals on animals and the environment (OECD, 2017). They are constantly supplemented and updated with new research methods. They contain internationally agreed test methods that are used to characterize the potential hazards of chemical substances. At the same time, it should be noted that in the practice of veterinary medicine in Ukraine today, there is no clearly described methodology for determining the toxicity of drugs for long-term dermal application. Today, the determination of subacute dermal toxicity of veterinary medicinal products is carried out according to the methodology (Kotsiumbas et al., 2006); however, it does not fully assess the toxicity of drugs.

*The research aimed* to determine the subacute toxicity of the drug for dermal application using the national (classical) method and the method according to OECD No. 410 (OECD, 1981). A wound-healing ointment, which includes betamethasone dipropionate, gentamicin sulfate, and clotrimazole, served as a marker for the comparative evaluation of the two methods.

## 2. Materials and methods

In the animals that were used in the comparative evaluation of the two methods, the day before the start of the experiment, hair was removed from the dorsal surface in an area not less than 10 % of the total surface area of the animal's body. Repeated hair removal was performed weekly. The product under study was applied to the prepared dorsal surface of the skin for 28 days. Determination of the subacute dermal toxicity of the studied agent was performed on healthy, young rats with intact skin and body weight 200–220 g.

When studying the subacute skin toxicity of the drug by the classical method, four groups of animals, five each, were formed according to the principle of analogs. Animals of the control group were given water on a pre-prepared area of the body; animals of the first experimental group were given the drug in a therapeutic dose (0.5 ml/kg body weight); animals of the second experimental group were given five times the

therapeutic dose (2.5 ml/kg of body weight); animals of the III experimental group – in ten times the therapeutic dose (5 ml/kg of body weight) (Kotsiumbas et al., 2006).

When studying the drug's effect on the organism of laboratory animals by the method described in the OECD No. 410, 4 groups of 5 animals each were formed according to the principle of analogs. The product under study was kept in contact with the skin for six hours using a porous gauze bandage. Animals of the control group were given water on a pre-prepared area of the body, animals of the first experimental group were given the drug under investigation at a dose of 0.5 ml/kg body weight, animals of the second experimental group were injected with a dose of 2.5 ml/kg body weight, animals III experimental group – at a dose of 5 ml/kg body weight. After the exposure period ended, the studied drug's remains were removed using water (OECD, 1981).

After the end of the experiment, the laboratory animals were decapitated under light ether anesthesia, blood was collected for hematological and biochemical studies, and the weight coefficients of the internal organs were determined. Blood stabilized with EDTA was used for hematological studies, and blood serum was used for biochemical studies. In the stabilized blood, the following were determined: hemoglobin content, number of erythrocytes and leukocytes, hematocrit, and red blood indices - with the help of a Mythic-18 hematological analyzer. In blood serum, the following were determined: total protein using an IRF-22 refractometer, enzyme activity (ALT, AST), creatinine content, and urea using a semi-automatic biochemical analyzer Humalyzer 3000 using standard sets of the Human company. The obtained data were processed statistically with the determination of average values, the credible interval at the available significance level of  $P < 0.05$ , taking into account the Student's criterion.

## 3. Results and discussion

When determining subacute skin toxicity using the classic method, it was found that 28-day use of the drug throughout the entire period of the experiment did not cause changes in the body weight of the animals and the weight coefficients of the liver, heart, spleen, kidneys, and lungs compared to the values of the animals in the control group (Table 1).

When determining the hematological indicators (Table 2), it was established that the use of the research product for 28 days in the animals of the first experimental group caused a slight decrease in the concentration of hemoglobin, the number of erythrocytes, leukocytes, hematocrit, and the average concentration of hemoglobin in the erythrocyte (MCHC) against the background of a slight increase in the average erythrocyte volume (MCV); the number of platelets compared to the indicators of the control group. In the animals of the II and III research groups, a tendency to increase hemoglobin concentration was noted. At the same time, no significant changes were detected in the number of erythrocytes, leukocytes, hematocrit, mean concentration of hemoglobin in erythrocytes (MCHC), mean volume of erythrocytes (MCV), mean content of hemoglobin in erythrocytes (MCH).

**Table 1**Weight coefficients of the mass of internal organs of white rats for the study of toxicity by the classical method ( $M \pm m$ ,  $n = 5$ )

Internal organs	Control	I group	II group	III group
Liver	33.6 ± 1.32	34.8 ± 1.55	32.1 ± 0.84	31.9 ± 0.99
Spleen	4.68 ± 0.24	4.56 ± 0.11	4.7 ± 0.21	4.74 ± 0.16
Heart	4.04 ± 0.21	4.24 ± 0.25	3.9 ± 0.21	4.24 ± 0.21
Lungs	13.1 ± 2.05	14.2 ± 3.45	11.2 ± 1.48	12.3 ± 1.23
Kidneys	7.64 ± 0.23	7.82 ± 0.33	7.58 ± 0.23	7.79 ± 0.26
Body weight	214.2 ± 4.76	218.4 ± 12.4	224.0 ± 7.13	222.0 ± 7.68

**Table 2**Hematological indicators of the blood of white rats for the study of toxicity by the classic method ( $M \pm m$ ,  $n = 5$ )

Indicators	Control	I group	II group	III group
Hemoglobin, g/l	155.4 ± 3.77	150.8 ± 2.03	157.4 ± 1.29	161.4 ± 4.35
Erythrocytes, T/l	7.49 ± 0.21	7.04 ± 0.17	7.25 ± 0.06	7.28 ± 0.17
Leukocytes, g/l	14.1 ± 1.71	12.4 ± 2.45	12.9 ± 1.18	13.5 ± 1.92
Hematocrit, %	42.3 ± 0.92	40.6 ± 0.74	41.5 ± 0.36	41.2 ± 0.78
MCH, pg	21.5 ± 0.12	21.5 ± 0.26	21.7 ± 0.09	22.2 ± 0.65
MCHC, g/dl	38.06 ± 0.55	37.1 ± 0.39	37.9 ± 0.18	39.3 ± 1.40
MCV, $\mu\text{m}^3$	56.6 ± 0.46	57.8 ± 0.98	57.2 ± 0.22	56.6 ± 0.73
Platelets	363.8 ± 20.9	398.4 ± 67.4	351.8 ± 55.5	417.2 ± 54.7

When determining the biochemical indicators of blood serum (Table 3), it was established that the dermal application of the drug in the animals of the 1st experimental group caused a decrease in the concentration of total protein and the level of creatinine, respectively, by - 5.9 and 1.9 %. In addition, a slight increase in the level of urea by 2.1% was established compared to the values of animals in the control group. When determining the activity of blood serum enzymes in the animals of the 1st experimental group, a tendency towards an increase in the activity of AST and ALT

was revealed, respectively, by - 4.2 and 2.1 %. While in the animals of the II and III research groups, the use of the studied agent caused a tendency to decrease the concentration of total protein, respectively, by - 5.5 and 6.8 %, the level of creatinine by - 3.1 and 5.3 %, and the activity of ALT - 6, 5 and 9.7 %. Under these conditions, there was an increase in the level of urea, respectively, by - 5.3 and 11.2 %, and activity of AST - by 9.8 and 14.9 % compared to the indicators of animals of the control group.

**Table 3**Biochemical indicators of the blood of white rats for the study of toxicity by the classical method ( $M \pm m$ ,  $n = 5$ )

Indicators	Control	I group	II group	III group
Total protein, g/l	77.9 ± 1.99	73.3 ± 1.26	73.6 ± 1.63	72.6 ± 2.38
Urea, mmol/l	8.72 ± 0.66	8.92 ± 0.42	9.18 ± 0.64	9.7 ± 0.37
Creatinine, $\mu\text{mol/l}$	77.0 ± 2.81	75.5 ± 3.45	74.6 ± 3.73	72.9 ± 4.17
AST, units/l	146.4 ± 6.59	152.5 ± 12.6	160.8 ± 11.7	168.2 ± 11.36
ALT, unit/l	82.6 ± 7.34	84.3 ± 10.4	77.2 ± 4.03	74.6 ± 7.16

Therefore, the study of the skin toxicity of the product under study by the classic method showed that in the animals of the experimental groups, long-term use of the drug did not cause significant changes in the concentration of hemoglobin, the number of erythrocytes, leukocytes, the value of hematocrit, the average concentration of hemoglobin in the erythrocyte (MCHC), the average volume of the erythrocyte (MCV), the average content of hemoglobin in the erythrocyte (MCH) and the number of platelets. At the same time, a decrease in total protein and creatinine content was noted, while the activity of AST and the level of urea increased.

When studying the effect of the drug on the organism of laboratory animals during the experiment, according to the methodology described in the OECD No. 410, it was established that the use of the researched agent throughout the entire period of the investigation in animals of the I, II and III experimental groups caused a probable decrease in the weight coefficients of the spleen mass, respectively, by -

19.1 ( $P < 0.05$ ), 22.4 ( $P < 0.05$ ) and 28.3 % ( $P < 0.05$ ), hearts - 3.6, 4.6 and 7.7 %, kidneys - 4.9, 6.5 and 10.4 % and animal body weight - 4.5, 5.4 and 6.4 %, and weight coefficients of liver weight probably increased by 10.8 ( $P < 0.05$ ), 11.8 ( $P < 0.05$ ) and 15.6 % ( $P < 0.01$ ). In addition, it should be noted that the use of the investigated agent did not cause significant changes in lung mass compared to the parameters of the control group of animals (Table 4).

When determining the effect of the researched agent on hematological parameters (Table 5) in animals of the I-III experimental groups, a decrease in hemoglobin concentration was established, respectively, by - 2.6, 2.9 and 4.4 %, the number of leukocytes - 21.4, 16.1 and 15.1 %, hematocrit values - 1.9, 3.6 and 4.0 %, average hemoglobin content in erythrocytes (MCH) - 8.3, 6.9 and 5.1 %, average hemoglobin concentration in erythrocytes (MCHC) - 1.7, 0.4 and 1.5 % and mean erythrocyte volume (MCV) - 5.03, 4.7 and 1.7 %.

When determining the biochemical indicators of blood serum (Table 6), it was established that the dermal application of the drug in animals of the 1st experimental group caused a decrease in total protein concentration by 5.8 %. At the same time, a slight increase in the level of creatinine, urea, ALT, and AST activity was noted, respectively, on – 6.2, 28.6 (P < 0.05), 3.8, and 14.7 % (P < 0.05).

In the animals of the II and III research groups, a probable increase in the level of urea was noted, respectively, by – 18.8 (P < 0.05) and 35.7 % (P < 0.05) and an increase in the level of creatinine – 8.4 and 3.5 %, AST activity – 16.4 (P < 0.05) and 11.3 %, and ALT activity – 8.5 and 11 %.

**Table 4**

Weight coefficients of the mass of internal organs of white rats (M ± m, n = 5)

Internal organs	Control	I group	II group	III group
Liver	31.04 ± 0.69	34.4 ± 1.08*	34.7 ± 1.31*	35.9 ± 1.25**
Spleen	5.08 ± 0.35	4.14 ± 0.17*	3.94 ± 0.08*	3.64 ± 0.35*
Heart	3.92 ± 0.17	3.78 ± 0.14	3.74 ± 0.11	3.62 ± 0.10
Lungs	7.58 ± 0.61	7.98 ± 0.49	8.08 ± 0.36	8.72 ± 1.42
Kidneys	7.72 ± 0.46	7.34 ± 0.38	7.22 ± 0.50	6.92 ± 0.28
Body weight	224.4 ± 6.14	214.4 ± 6.62	212.2 ± 4.15	210.0 ± 4.15

Note: \* – P < 0.05, \*\* – P < 0.01

**Table 5**

Hematological indicators of the blood of white rats (M ± m, n = 5)

Indicators	Control	I group	II group	III group
Hemoglobin, g/l	154.2 ± 6.13	150.2 ± 2.42	149.8 ± 5.72	147.4 ± 3.14
Erythrocytes, T/l	7.32 ± 0.19	7.55 ± 0.14	7.39 ± 0.11	7.15 ± 0.16
Leukocytes, g/l	9.82 ± 1.18	7.72 ± 1.64	8.24 ± 0.93	8.34 ± 1.27
Hematocrit, %	42.1 ± 0.97	41.3 ± 0.74	40.6 ± 0.75	40.4 ± 0.70
MCH, pg	21.7 ± 0.51	19.9 ± 0.24*	20.2 ± 0.48	20.6 ± 0.14
MCHC, g/dl	37.04 ± 0.34	36.4 ± 0.31	36.86 ± 0.88	36.5 ± 0.25
MCV, μm <sup>3</sup>	57.6 ± 1.26	54.7 ± 0.72	54.9 ± 0.67	56.6 ± 0.32
Platelets	610 ± 85.0	598.0 ± 44.5	549.3 ± 56.6	618.2 ± 54.7

**Table 6**

Biochemical indicators of the blood of white rats (M ± m, n = 5)

Indicators	Control	I group	II group	III group
Total protein, g/l	76.9 ± 3.17	72.4 ± 3.08	74.9 ± 2.18	84.1 ± 1.57
Urea, mmol/l	8.4 ± 0.39	9.98 ± 0.49*	10.8 ± 0.28***	11.4 ± 0.27***
Creatinine, μmol/l	63.4 ± 5.31	67.3 ± 3.99	68.7 ± 5.15	65.6 ± 6.05
AST, units/l	181.5 ± 6.51	208.1 ± 8.39*	211.2 ± 6.54*	201.9 ± 12.2
ALT, unit/l	74.5 ± 4.51	77.3 ± 3.56	80.8 ± 0.90	82.7 ± 4.90

Note: \* – P < 0.05, \*\* – P < 0.01

Therefore, when studying the subacute dermal toxicity of the tested agent according to the OECD method No. 410, it was established that 28-day use of the drug caused a decrease in the weight coefficients of the spleen mass, hemoglobin concentration, the number of leukocytes, the hematocrit value; the average hemoglobin content in the erythrocyte (MCH), the average concentration hemoglobin in the erythrocyte (MCHC) and the average volume of the erythrocyte (MCV), which probably indicates inhibition of hemopoietic processes. In addition, a decrease in the weight coefficients of the heart, kidney, and body weight of animals was noted against the background of an increase in the weight coefficients of the liver mass, urea level, creatinine, AST, and ALT activity, which probably indicates a liver function disorder (damage to the cytoplasm of liver cells, destructive processes in cells liver) and kidney.

#### 4. Conclusions

The data of experimental studies indicate that the results obtained in the experiment according to the methodology of the OECD No. 410 compared with the classical method provide more information about the toxic effect of the investigated agent on the animal body. In particular, a decrease in the weight coefficients of the spleen, heart, kidney, animal body weight, hemoglobin concentration, leukocyte count, hematocrit value, average hemoglobin content in erythrocytes (MCH), average erythrocyte volume (MCV) was found in animals. Under these conditions, the weight coefficients of liver weight, number of erythrocytes, level of urea, creatinine, and activity of AST and ALT were noted.

In further studies, it is advisable to study the toxicity of different pharmacological groups of veterinary medicines in a comparative aspect.

### Conflict of interest

The authors declare that there is no conflict of interest.

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