Article

Preclinical studies of natural astaxanthin in laboratory animals.

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Abstract: This article discusses the issue of finding new dental drugs that have antibacterial, anti-inflammatory, wound healing, immunomodulatory effects and exhibit minimal side effects to avoid complications from internal organs.

Keywords: antioxidant gel, astaxanthin, interferon, acute and chronic toxicity, local irritant effect, preclinical studies.

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1. Introduction

The search for new promising dental products with anti-inflammatory, wound healing, antibacterial and immunomodulatory effects and thus showing minimal side effects is a rather urgent task of modern scientific and practical medicine. Astaxanthin is a natural antioxidant that is present in various quantities in living organisms and belongs to the class of carotenoids by its chemical structure.

Another active ingredient of dental gel is interferon alpha, which has the ability to stimulate phagocytic activity of macrophages and cytotoxic activity of T-cells and NK-cells. It has an indirect antiviral effect, increasing the body's resistance to viral infections and modulating the immune system response aimed at neutralizing viruses or destroying cells infected by them.

2. Patients and Methods

Antioxidant gel with interferon was studied in young outbred rats.

3. Results

According to the results of the experimental study there were no changes in the clinical and biochemical analysis of peripheral blood in both experimental and control groups of animals. No significant changes in the behavior and motor activity of the animals of the experimental groups were observed after 30-day administration of the gel. Rectal temperature measurement data demonstrate that in animals of both the experimental and control groups the body temperature did not differ from normal. No signs of local inflammatory reaction were registered during application of the studied gel to the mucous membranes of the oral cavity.

4. Conclusion.

In the study of toxicity and possible local irritant effects no complications in the internal organs and no local inflammatory reaction were registered, as evidenced by the results of experimental studies.

5. Authors' Contributions.

Currently, the problem of development and introduction into dental practice of modern, effective and safe in the use of medicines based on active pharmaceutical substances of domestic origin is a rather pressing problem of medical science and practice.

When creating a dental gel with antiviral and anti-inflammatory properties, it was decided to use astaxanthin and interferon-alpha as the main active substances.

Astaxanthin is a carotenoid belonging to the group of xanthophylls. The chemical structure of this compound provides it with a pronounced antioxidant activity that makes it stand out among other natural antioxidants [1]. At present astaxanthin is successfully used in medical practice [2].

Another active ingredient of the dental gel is interferon alfa. It has the ability to stimulate the phagocytic activity of macrophages as well as the cytotoxic activity of T cells and NK cells, has an indirect antiviral effect, increasing resistance to viral infections and modulating the immune system response to neutralize viruses or destroy cells infected by them [9,10].

Currently, the urgent task of medicine is to develop domestic remedies that not only have antiviral effect, anti-inflammatory, wound healing, antibacterial and immunomodulatory properties, but also have no side effects, as well as not causing addiction [11].

This study aims to establish the toxic, maximum tolerated and lethal doses of the developed dental gel and to study its effect on the main systems and functions of the body when administered intragastrically (in a single injection) and when applied locally (in a chronic toxicity study) in the studied doses to mature animals compared with placebo in accordance with the existing requirements.

The study is part of the complex of preclinical studies of the developed drug.

6. Purpose of work.

To carry out toxicity studies on laboratory animals of a dental gel (Astadent, gel for external use 2.6 % with interferon) containing natural astaxanthin and interferon alpha as active pharmaceutical substances.

7. Materials and methods.

The object of the study was the developed Astadent gel containing natural astaxanthin and interferon alpha as active pharmaceutical substances.

Determination of acute toxicity was performed on outbred mice (males, 66 animals) with a single injection of 500 mg/kg. Clinical signs of intoxication (lethargy, ruffling, stunned, shortened breathing, salivation) were revealed.

The study drug was administered intragastrically with a special probe in increasing doses. Dosing was carried out based on the content of the active substances. The follow-up period was 14 days.

During the entire experimental period, each animal was observed twice a day: in the morning and in the afternoon. On the day of drug administration, the animals were observed every hour. The results of the examinations were recorded in laboratory charts.

Assessment of the toxic effect of Astadent gel was carried out according to the following clinical signs:

- number and timing of animal death (if any);
- respiratory indicators (labored breathing, cyanosis, rapid breathing, nasal discharge);
- motor activity (elevated/reduced, somnolence, loss of balance, sensitivity, catalepsy, ataxia, unusual movements, prostration, tremor, fasciation);
- convulsions (clonic, tonic, tonic-clonic, asphyxial);

reflexes (corneal, equilibrium, myotactic, light, fright reflex);

-eye signs (lacrimation, miosis, mydriasis, exophthalmus, ptosis, clouding, iritis, conjunctivitis, chromodactria, weakening of the blinking membrane);

-high salivation;

-condition of hair coat (piloleiomyoma, alopecia);

-muscle tone (hypotension, hypertension);

- indicators of the gastrointestinal tract (soft stool, diarrhea, vomiting, polyuria);

-dynamics of body weight;

-macroscopic examination of organs and tissues;

-morphometric evaluation of organs (heart, thymus, liver, kidneys, adrenal glands, lungs, spleen, testes, ovaries).

To assess chronic toxicity, the drug was applied to the gingiva for 30 days. The surface of the treated mucosa was approximately 0.5x0.5 cm. In this regard, it was decided to use 2 doses when the drug was repeatedly applied:

- 43 mg/kg - approximately 10 times the maximum daily dose for humans per rat -8.6 mg/rat.

- 86 mg/kg - about 100 times the maximum human daily dose per rat - 17.2 mg/rat.

The dose of interferon was 106 IU -14286 IU/kg.

The studies were performed on young male outbred rats, which were randomly allocated groups (5 animals in each group, males/females). The first group received prophylactic gel for 30 days at a dosage of 43 mg/kg animal weight, the second group received 86 mg/kg, the third group being the control did not receive Astadent gel. The initial criterion was the body weight of the animal, which was 180-200 g.

In the subchronic experiment, animals were observed once a day, immediately before the application of the drug, to detect deviations in health status and mortality. The appearance of the animals in the cage, the behavior of each, the state of feces, etc., were examined. Animal body weight was determined weekly before administration of the study drug. Feed and water consumption were recorded daily.

Thirty days after drug administration all animals of the group (preliminarily deprived of food for the night) had biochemical and clinical blood parameters determined. Blood sampling was performed from the tail vein.

To determine clinical parameters, blood was placed in 0.9 ml tubes with EDTA and tested on an automatic hematology analyzer PCE 90 VETHTI, ERMA (Japan) to determine the number of erythrocytes, leukocytes, thrombocytes, hemoglobin level, hematocrit, etc.

To assess biochemical parameters, blood was collected in 1.0-2.0 ml tubes without anticoagulant, centrifuged to obtain serum, in which the following parameters were determined on automatic biochemical blood analyzer ILAB 650 (USA) using kits from "Biosistems", Spain: total protein, albumin, total cholesterol, triglycerides, total bilirubin, glucose, urea, creatinine, alkaline phosphatase activity, alanine and aspartataminotransferase.

8. Statistical analysis.

After completing the experimental studies, statistical processing of the results was carried out by the method of variation statistics using Student's t-criterion. Statistical data were processed using Microsoft Office Excel.

9. Results and discussion.

9.1. Clinical observations.

Application of Astadent in animals for 30 days at a dose of 43 mg/kg and 86 mg/kg, respectively, did not cause changes in the main integral indices in rats.

The animals were active and had a neat appearance. The consumption of dry feed and water by the rats of the experimental groups corresponded to the control parameters. During the subchronic experiment, no animal death was observed in any of the experimental groups.

9.2 Effect on body weight, feed and water consumption

During the experiment, the test animals were monitored, recording the change in body weight weekly. The dynamics of weight changes for female and male rats for 30 days are shown in Table 1 & Table 2.

Table 1. Change in body weight in white male rats during the course of application of "Astadent" gel.

Drug, dose	Changes in the body weight of animals as a % of the initial through:			
	1 day		30 days	
	m	Sr	m	Sr
Control	119,5	5,0	119,2	5,4
Test drug "Astadent"				
43 mg/kg	121,2	4,1	121,5	4,1
86 mg/kg	122,5	3,1	122,1	4,1

Table 2. Change in body weight in female white rats during the course of application of "Astadent" gel:

Drug, dose	Changes in the body weight of animals as a % of the initial through:			
	1 day		30 days	
	m	Sr	m	Sr
Control	116,5	4,1	120,6	3,1
Test drug "Astadent"				
43 mg/kg	119,4	3,2	119,5	4,1
86 mg/kg	119,8	4,0	119,5	4,1

In the groups that received Astadent dental gel in both doses, the body weight gain did not differ from the weight gain of control animals.

The daily feed and water consumption was counted weekly. The amount of feed and water consumption by the rats receiving the above preparations did not statistically differ from the parameters of the animals in the control groups (Table 3).

Table 3. Daily water and feed intake in white rats after application of «Astadent» gel:

Drug, dose	Males				Females			
	Dry feed intake, g /100 g		Water consumption, ml /100 g		Dry feed intake, g /100 g		Water consumption, ml /100 g	
	m	Sr	m	Sr	m	Sr	m	Sr
Control	8,54	1,30	8,30	1,30	6,40	1,20	7,20	1,37
Test drug "Astadent"								
43 mg/kg	9,15	1,10	9,70	1,10	6,25	1,70	9,10	1,30
86 mg/kg	9,10	1,20	9,50	1,19	7,55	2,00	9,10	1,20

9.3 Effect on animal body temperature:

The body temperature of the animals was measured rectally. The state of these vital signs of the body was studied in accordance with the Protocol at the beginning and 30 days after the beginning of the application. The results of measuring the rectal temperature of the rats (5 animals of each sex from the experimental groups) using an electric medical thermometer TPM - 1 (permissible basic error from the range of measured temperatures, % ± 1) are presented in Table 4.

Table 4. Effect of astadent gel on the rectal temperature of white rats (oC, Mm)

Timing of the study	"Astadent», mg			
	Control		86 mg/kg	
	M	F	M	F
Background	34.1±0.2	35.2±0.1	35.1±0.2	35.1±0.2
30 days	35.2±0.2	35.3±0.1	35.1±0.2	35.3±0.2

Rectal temperature measurement data demonstrate that in the animals of the experimental groups, as well as in the control group, the body temperature did not differ from the baseline data.

9.4 Influence on parameters of functional state of kidneys.

Condition of excretory system - kidneys was determined after water load, which was 2.5 % of "starvation" body weight. Eighteen hours before the experiment the animals were deprived of

food, leaving free access to water, consistently for four hours the animals were placed in exchange chambers to collect urine. The volume of excreted urine was measured, diuresis per 100 g of each animal's weight was calculated, and the relative density of urine was determined (Table 4 & Table 5)

Table 4. Urine parameters of male rats when using «Astadent» gel

Drug, dose	Urine volume / relative urine density	Presence of pathological elements in the urine
Control	1,71/6,40	-
Test drug "Astadent"		
43 mg/kg	1,75/6,3	-
86 mg/kg	1,77/7,2	-

Table 5. Urine parameters of female rats when using «Astadent» gel

Drug, dose	Urine volume / relative urine density	Presence of pathological elements in the urine
Control	1,72/6,1	-
Test drug "Astadent"		
43 mg/kg	1,74/6,2	-
86 mg/kg	1,75/6,1	-

9.5 Effect on motor and exploratory activity.

Experiments were performed on 5 rats of each sex from each experimental group. The rats were placed one by one in the "open field" system, where their movements were recorded for 35 minutes. Table 6 & Table 7 presents the data on the effect of astadent gel on the spontaneous motor activity (SDA) of the rats.

Table 6. Effect of astadent gel on motor activity of rats of white rats (male)

Drug, dose	Number of crossed squares		Number of stand ups		Number of peeks into the holes
Control	9,3		3,2		8,2
Test drug "Astadent"					
43 mg/kg	10,3	1	3,0	1	8,0
86 mg/kg	9,2	1	3,1	1	8,1

Table 7. Effect of astadent gel on motor activity of rats of white rats (females)

Drug, dose	Number of crossed squares		Number of stand ups		Number of peeks into the holes
Control	10,2		3,1		8,1
Test drug "Astadent"					
43 mg/kg	10,2		3,2		8,2
86 mg/kg	10,3		3,2		8,1

The data given in the tables indicate that there were no significant changes in the structure of behavior and motor activity of the animals of the experimental groups after 30 days of gel administration. A change in the behavioral pattern, characteristic of the animals secondary placed in the "open field" experiment, was noted, mainly due to the lengthening of the latent period, which was also of an unreliable nature.

9.6 Effect on peripheral blood parameters.

Peripheral blood parameters in animals treated with astadent gel are presented in Table 8 & Table 9.

Table 8. Effect of the test Astadent gel on the composition of peripheral blood in male white rats, (M ±m) (after 30 days)

The studied indicators	Control	"Astadent" 43 mg/kg	"Astadent" 86 mg/kg
Leukocytes, *10^9/l	7,8±0,1	7,5±0,1	8,2±0,1
Erythrocytes,*10^{12}/ l	5,8±0,1	6,9±0,1	6,4±0,1
Hemoglobin, g/ l	137,0±0,1	125,5±0,2	140,0±0,1
Hematocrit, %	33,0±0,2	38,8±0,2	35,4±0,2
MCV, Fl.	50,0±0,1	45,0±0,2	47,8±0,2
MCH, picograms	21,0±0,1	20,0±0,1	19,0±0,1
MCHC, g/l	30,0±0,2	32,6±0,1	31,8±0,2
Platelets, *10^9/l	569,0±0,2	593,3±0,2	544,0±0,2
RDW, %	18,0±0,1	18,0±0,1	17,9±0,1
Lymphocytes, %	74,0±0,2	77,0±0,1	65,0±0,2
Monocytes, %	4,0±0,1	3,8±0,1	4,9±0,1
Eosinophils, %	0,5±0,01	0,3±0,01	0,6±0,01
Bacillary, %	0,5±0,02	0,5±0,01	0,4±0,01
Segmentonuclear, %	21,0±0,1	18,4±0,1	29,1±0,1

Table 9. Effect of the test Astadent gel on peripheral blood composition in female white rats, (M ±m) (after 30 days).

The studied indicators	Control	"Astadent" 43 mg/kg	"Astadent" 86 mg/kg
Leukocytes, *10^9/l	7,7±0,1	7,9±0,1	8,3±0,1
Erythrocytes,*10^{12}/ l	6,0±0,1	6,5±0,1	5,0±0,1
Hemoglobin, g/ l	140,0±0,2	138,0±0,2	136,0±0,1
Hematocrit, %	35,0±0,2	37,0±0,2	32,5±0,2
MCV, Fl.	49,0±0,2	50,4±0,2	58,0±0,2
MCH, picograms	22,0±0,2	26,0±0,2	19,0±0,1
MCHC, g/l	30,0±0,2	41,0±0,3	39,0±0,2
Platelets, *10^9/l	590,0±0,1	615,0±0,2	589,0±0,2
RDW, %	18,0±0,1	23,0±0,1	20,0±0,1

Lymphocytes, %	69,0±0,1	73,0±0,1	65,0±0,2
Monocytes, %	5,0±0,1	3,0±0,1	7,0±0,1
Eosinophils, %	0,9±0,01	0,5±0,01	0,5±0,01
Bacillary, %	0,1±0,01	0,7±0,01	0,6±0,01
Segmentonuclear, %	25,0±0,1	22,8±0,2	26,9±0,1

Tabl. 9

The studies showed that lymphocytes have pycnotized nucleus, granularity is detected. The main mass of segmentonuclear cells has gentle, neutrophilic granularity in smears. Eosinophilic cell nuclei are formed of loose chromatin substance and are almost circular in shape. Monocytes are very different from lymphocytes, equal in size to two erythrocytes, have a large bean-shaped nucleus and a broad protoplasmic border, which is stained blue or purple with delicate granulation. Blood plates lie in large clumps. There were no significant differences between the parameters of the control and experimental groups. No sex differences were found.

Thus, the peripheral blood of rats of all experimental groups after 30 days of application of the test preparation "Astadent" by its quantitative and qualitative composition corresponded to the species physiological norm.

9.7 Influence on biochemical blood parameters.

Table 10 presents the data on the effect of the test drug "Astadent" on the main biochemical indices and on the enzyme activity and blood ion balance of white rats. The study was performed on a biochemical automatic analyzer ILAB 650 (USA).

Table 10. Effect of the test Astadent gel on the main biochemical indices of peripheral blood of white rats, (M ± m).

Indicator	Control	"Astadent" 43 mg/kg	"Astadent" 86 mg/kg
Total bilirubin (BilT), μmol/L	2,2±0,1	2,0±0,2	1,80±0,2
AST, E/l	620,0±0,1	65,3±0,2	64,8±0,2
Creatinine (CREAT)	62,0±0,1	61,8±0,2	63,0±0,2
ALT, E/l	71,2±0,1	71,4±0,1	72,0±0,2
Alkaline phosphatase (ALP), E/l	131,3±0,2	127,5±0,2	135,0±0,2
Glucose (Glu), mmol/l	4,7±0,1	4,5±0,1	4,8±0,1
GGT, E/l	111,5±0,1	112,0±0,1	108,5±0,1
Amylase (AMYL), E/l	641,0±0,1	614,2±0,1	642,0±0,1
Total protein (T PROT), g/l	61,7±0,1	61,9±0,1	64,3±0,1
KFK, E/l	85,0±0,1	86,5±0,1	87,7±0,1
Urea (UREA), mmol/l	5,4±0,1	5,7±0,1	5,9±0,1

As can be seen from the data presented in the table, both the studied gel and the comparison drug in both tested doses have no adverse effect on the main biochemical blood parameters, the activity of blood plasma enzymes and its electrolyte balance.

9.8 Study of possible local irritation

No signs of local inflammatory reaction (infiltration, redness) were registered when applying the studied Astadent gel to the oral mucosa, which was confirmed by visual and histological examination.

The results obtained are presented in Table 11.

Table 11. Registration of local inflammatory response in rats after application of «Astadent» gel

Dose per active ingredient, mg/kg	43	86
1 day		
«Astadent»	0	0
7 day		
«Astadent»	0	0
14 day		
«Astadent»	0	0

10. Conclusion.

In the study of toxicity and possible local irritant effects no complications in the internal organs and local inflammatory response were recorded.

In acute toxicity studies on mice it was shown that the LD50 of the test gel "Astadent" was not established. Subsequent observation of the animals showed no deviations in the appearance, condition of the coat and mucous membranes, character of excretions, behavioral reactions, weight gain.

When applying "Astadent" gel to the mucous membranes of the tested animals, no differences in the manifestation of toxic effects were also noted. Throughout the experiment on evaluation of chronic toxicity in animals treated with "Astadent" gel, no deviations in motor activity were detected. The state of skin, mucous membranes and hair remained normal. The dynamics of changes in the weight of female and male rats during 30 days did not differ from the weight of control animals. The amount of feed and water consumed by the rats receiving the studied preparation did not statistically differ from the parameters of the animals in the control groups.

Orientational motor activity, as an indicator of the central nervous system state, in rats of both sexes that received the test gel with astaxanthin produced by (PFUR, RF) Center for Collective Use (Scientific and Educational Center) of PFUR did not differ from the same indicator in control animals.

The gel had no adverse effects on the peripheral blood parameters and biochemical blood parameters, detoxifying function of the liver.

Conflicts of Interest: The authors declare no conflict of interest.

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