TRANSLATED FROM RUSSIAN

CHRONIC EXPERIMENT

1. The chronic experiment included the following preclinical trials:

1.1. The chronic toxicity of the material was studied in accordance with GOST R ISO 10993.11-99 “Medical devices. Study of biological action of medical devices. Part 11. Research of general toxic action”.

1.2. The local action of the material after the implantation (implantation test) was studied in accordance with GOST R ISO 10993.10-99 “Medical devices. Study of biological action of medical devices. Part 6. Research of the local action after implantation”.

1.3. The gonadotoxic action of the material was studied in accordance with GOST R ISO 10993.10-99 “Medical devices. Study of biological action of medical devices. Part 3. Research of genotoxicity, carcinogenicity and toxic action on the reproductive function” by the condition of the histological structure of male organs of reproduction.

1.4. The pathomorphological study of the experimental animals’ internals was conducted in accordance with GOST R ISO 10993.10-99 “Medical devices. Study of biological action of medical devices. Part 3. Research of genotoxicity, carcinogenicity and toxic action on the reproductive function”.

The experimental animals were kept in accordance with GOST R ISO 10993.2-99 “Medical devices. Study of biological action of medical devices. Part 2. Regulations on protection of animals”.

Samples. The material is submitted in a sterile form. “ARGIFORM” is filled in disposable injection plastic syringes, which are tipped and vacuum-sealed in individual blister packaging. Each set consisting of a blister packaging with a syringe and an injection cannula which are packed into a separate branded carton box. The marking, description and the trademark are printed on the boxes.

2. Description and results of the experiment.

2.1. Description of the experiment.

The chronic experiment was conducted on 12 outbred dogs. To 9 of them the material “ARGIFORM” was implanted subcutaneously by injection at hip in quantity of 15 ml. And 3 control dogs received a control (false) introduction.
The observation of animals lasted for 6, 12 and 18 months.
All animals were examined before the experiment and then in 6, 12 and 18 months

after the implantation. The following hematological indices were determined: (quantity of red blood cells, leukocytes, thrombocytes, hemoglobin level), as well as certain biochemical indices and activity of blood serum ferments (total protein, triglycerides, cholesterol, bilirubin, creatinine, urea, glucose, activity of alkaline phosphatase, of lactate dehydrogenase, of aspartate and alanine aminotransferases). In order to research the above indices, some blood from saphenous veins of dogs' shins was drawn.
During the experiment general condition and behaviour of animals was observed, the body mass and rectal temperature were registered.
Blood count was made by means of an automatic counter: hematological analyzer PICOSCALE PS-4 (Hungary).

The hemoglobin level was determined by the hemoglobin cyanide method.

Total amount of serum protein, creatinine, urea and bilirubin, as well as an activity of lactate dehydrogenase and alkaline phosphatase were determined with the help of special laboratory kits of "Diacom Synteco" (Russia).

The determination of the level of triglycerides and cholesterol amount was carried out using the DiaSys set (DiaSys Diagnostic Systems GmbH, Germany).

The aspartate aminotransferase and alanine aminotransferase activity was determined with the help of special laboratory sets of "Corway" (Russia).

A special laboratory kit of "Ani Labsystems Ltd." (Finland) was used for detection of serum glucose level.

The universal control serums named "Norm" and "Pathology" (DiaSys Diagnostic Systems GmbH, Germany) were applied into the biochemical tests.

Biochemical indices and activity of ferments of serum blood of tested animals were determined using Labsystems biochemical analyzer FP-901 (Finland).

At last point of experiment, dogs were euthanized by thiopental and droperidol overdosing injection, animal internals were subjected to a macroscopic and histological study with the use of above-mentioned methods (p.5.2.3.1.).

2.2. Results of the study

The results are shown in tables ##1-8 as M±m, where M is the arithmetic average of the results of measurement, and m is the standard deviation of the results of measurement.

Table #1

Dynamics of the dogs' body mass, % from the initial mass

Animal groups	Periods of observation		
	6 months	12 months	18 months
Control	102.00±1.76	103.76±1.54	105.80±1.05
Experiment	101.72±1.04	103.21±1.21	105.04±0.85
P	> 0.05	> 0.05	> 0.05

The measurements of temperature did not show any differences of the indices of dogs from experimental and control groups during the whole period of study (table #2).

Table #2

Indices of the dogs' rectal temperature, °C

Animal groups	Periods of observation			
	1 month	6 months	12 months	18 months
Control	38.86±1.36	38.53±0.980	38.8±0.96	38.33±1.53
Experiment	38.24±1.33	38.12±0.95	38.52±0.95	38.24±1.35
P	> 0.05	> 0.05	> 0.05	> 0.05

During the study of the morphological composition of the periphery blood of experimental dogs no reliable differences of hematological indices were observed in comparison with the control ones (table #3).

Table #3
Hematological indices of the dogs during the subcutaneous material implantation of 18 months duration

Periods of observation	Animal groups		R
	Control	Experiment	
	Red blood cells, 10^{12}/l		
Before the injection (history)	6.76±0.37	6.55±0.23	> 0.05
6 months	7.06±0.49	7.15±0.24	> 0.05
12 months	6.93±0.43	6.91±0.23	> 0.05
18 months	7.16±0.39	7.14±0.25	> 0.05
	Leukocytes, 10^{9}/l		
Before the injection	10.96±0.58	10.37±0.44	> 0.05
6 months	10.93±0.48	10.40±0.63	> 0.05
12 months	10.80±0.55	11.04±0.45	> 0.05
18 months	10.73±0.47	11.13±0.55	> 0.05
	Thrombocytes, 10^{9}/l		
Before the injection	411.00±20.03	403.88±18.46	> 0.05
6 months	399.00±18.77	406.11±17.5	> 0.05
12 months	409.66±17.40	391.33±18.40	> 0.05
18 months	407.00±17.61	411.77±19.60	> 0.05
	Hemoglobin, g/l		
Before the injection	107.33±5.36	104.66±6.50	> 0.05
6 months	108.33±4.41	106.00±4.42	> 0.05
12 months	109.66±6.96	107.77±5.56	> 0.05
18 months	108.33±6.36	109.22±4.51	> 0.05

During 18 months of the chronic experiment on subcutaneous implantation the material did not exert any influence on levels of serum total protein at experimental animals. This confirms an absence of material damaging effect upon the protein-forming function of liver (table #4).

Table #4
The blood serum total protein of dogs during subcutaneous implantation of the material of 18 months duration, %

Periods of observation	Control	Experiment	P
Before the injection (history)	83.83±5.58	78.25±4.76	> 0.05
6 months	89.26±6.02	81.70±4.46	> 0.05
12 months	89.90±6.67	87.62±9.47	> 0.05
18 months	82.00±6.76	83.60±4.71	> 0.05

In order to reveal a possible damaging action of the material during chronic experiment on the function of the dogs' liver, the activity of alkaline phosphatase, lactate dehydrogenase, aspartate and alanine aminotransferases was studied, as well as the level of bilirubin in the blood serum of the animals.
As the conducted study showed, the presence of the material in the animals did not cause any changes of the activity of the above mentioned 'hepatic' ferments and the level of bilirubin in the blood serum of the experimental dogs (table #5).

Table #5
Ferment activity indices and blood serum bilirubin quantity of dogs during the material subcutaneous implantation.

Periods of observation	Groups of animals		
	Control	Experiment	P
	Alkaline phosphatase, U/l		
Before the injection (history)	159.00±14.96	156.50±11.38	> 0.05
6 months	181.23±16.18	193.48±22.54	> 0.05
12 months	188.46±19.12	165.74±17.96	> 0.05
18 months	200.66±17.26	186.81±14.74	> 0.05
	Alanine aminotransferase, U/l		
Before the injection	40.90±4.10	42.47±1.82	> 0.05
6 months	51.76±3.96	51.55±2.44	> 0.05
12 months	48.96±3.62	53.71±1.93	> 0.05
18 months	52.83±4.74	46.00±1.89	> 0.05
	Aspartate aminotransferase, U/l		
Before the injection	23.10±1.96	27.71±1.10	> 0.05
6 months	30.43±3.46	26.83±1.04	> 0.05
12 months	27.16±1.90	23.26±0.90	> 0.05
18 months	26.06±1.84	24.57±0.88	> 0.05
	Lactate dehydrogenase, U/l		
Before the injection	206.66±11.87	214.54±8.44	> 0.05
6 months	200.60±15.83	179.83±7.40	> 0.05
12 months	189.06±14.59	193.54±6.37	> 0.05
18 months	212.30±15.07	196.28±7.43	> 0.05
	Bilirubin, µmol/l		
Before the injection	10.95±0.96	9.27±0.36	> 0.05
6 months	9.79±0.77	10.52±0.36	> 0.05
12 months	9.51±0.38	10.78±0.34	> 0.05
18 months	10.56±0.64	9.93±0.34	> 0.05

During the 18 months chronic experiment in the blood serum of the experimental dogs no changes of the levels of urea and creatinine were observed so that carries inference about absence of material's damaging action on the excretory function of the kidneys of the experimental animals (table #6).

Table #6
Levels of urea and creatinine in the blood serum of the dogs.

Periods of observation	Animal groups		
	Control	Experiment	P
	Urea, mmol/l		
Before the injection (history)	6.06±0.44	6.85±0.25	> 0.05
6 months	6.96±0.50	6.25±0.25	> 0.05
12 months	6.56±0.43	5.66±0.22	> 0.05
18 months	6.70±0.30	5.76±0.23	> 0.05
	Creatinin, mmol/l		
Before the injection	136.16±5.48	140.34±3.35	> 0.05
6 months	144.90±4.57	140.55±5.50	> 0.05
12 months	141.26±5.93	147.20±5.65	> 0.05
18 months	148.96±3.87	142.67±3.94	> 0.05

For determination of influence of a long-term material implantation to the carbohydrate metabolism and to the function of dogs' pancreas, glucose blood serum level was determined. As this study results showed, the glucose level of animals in experimental group has not changed during the whole experiment and corresponded to the standard values characteristic of dogs (table #7).

Table #7
Glucose blood serum level in mmol/l of dogs after material subcutaneous implantation.

Periods of observation	Animal groups		
	Control	Experiment	P
Before the injection (history)	4.93±0.29	4.65±0.15	> 0.05
6 months	4.53±0.33	4.42±0.14	> 0.05
12 months	5.03±0.23	4.43±0.14	> 0.05
18 months	5.00±0.36	4.57±0.15	> 0.05

To study a possible damaging effect of the material on the lipid metabolism the total cholesterol and triglyceride levels were measured.

As the conducted study showed, the subcutaneous implantation of the material did not affect mentioned indices of lipid metabolism (table #8).

Table #8
Blood serum level of cholesterol and triglycerides of dogs after material subcutaneous implantation.

Periods of observation	Animal groups		
	Control	Experiment	P
	Triglycerides, mmol/l		
Before the injection (history)	0.50±0.04	0.45±0.01	> 0.05
6 months	0.49±0.04	0.58±0.02	> 0.05
12 months	0.47±0.04	0.47±0.01	> 0.05
18 months	0.56±0.02	0.52±0.01	> 0.05
	Total cholesterol, mmol/l		
Before the injection	3.81±0.35	3.32±0.14	> 0.05
6 months	3.93±0.38	4.21±0.17	> 0.05
12 months	4.21±0.25	3.63±0.13	> 0.05
18 months	4.34±0.35	3.92±0.15	> 0.05

Thus, the results showed that the presence of material “ARGIFORM” in dogs’ tissues during 18 months has not influenced their main habitus and behaviour and has not changed the functional status of the most important internals and systems of the animal organism.

2.3. Pathomorphological study

2.3.1. Study of organs

A macroscopic examination conducted in both groups (the control and the experimental one) and did not reveal any differences in the animals’ organs.

In both groups there were dogs with thick hair, moderately nutritional state; mucous membranes are humid with marked cyanotic tint. Cutaneous integuments and body skeleton are not damaged. The localization of the internals is usual.

The serous membranes of cardiac cavity are smooth, humid, grey and pellicle-like. Veins and venous sinuses are filled with a dark and liquid blood.

The margin between grey and white matter of the cerebrum and the spinal cord are clear-cut, the tissue is bright in places of sections, and there are no nidal changes.
The ventricles are slit-like, with grey flaccid vascular venous plexuses and a small amount of transparent liquid.

The hypophysis is rounded, encapsulated, flaccid, pale grey.
Both of the thyroid gland lobes are located symmetrically, they are blood-filled, reddish, fine-grained.

The salivary glands are encapsulated, compact, grey and lobulous.

The thymic gland is of a cyanotic colour, lobated with confluent ecchymoses under the capsule.

The spleen is with a smooth capsule, compact, the pulp is red, without a scrape.
The liver is compact, with a smooth capsule, blood-filled, red and brown.

The biliary ducts are permeable, walls of the gall bladder are thin, its mucous membrane is velvety, the bile in the gall bladder cavity is dark olive-green.

The pancreas is in a capsule, dense, lobated and grey.

The adrenals are of an oval shape, lobated, with a clear-cut division of the parenchyma into cerebral and cortical layer.

The kidneys are compact, with a smooth surface, with a capsule which is easily taken off, with a precise margin of between a reddish cortex and a pale grey cerebral layer, the mucous membranes of renal pelvises and of the urinary bladder are smooth.

In the trachea and main bronchial tubes there are some mucus, their mucous membrane is smooth and brilliant.

Lungs are air-filled, covered with grey thin pleura, the antero-inferior lobes are filled with blood irregularly.

The heart is hard, with smooth semiopaque valves and a smooth endocardium, and a grey and red, succulent, without nidal changes, compact myocardium, a moderate quantity of ecchymoses under the epicardium and with a usual pattern of the location of coronary arteries and veins.

An intima of the tube of pulmonary artery and aorta are smooth and brilliant.

The oesophagus is passable; its mucosa is smooth, whitish, hypertrophic.

The stomach is corn-shaped, with thin walls and relief grey mucosa folds, without any nidal changes.

The intestines contain a chymus coloured with gall and brown gruel-like faeces. The walls of intestines are thin, the mucous membrane of the small intestine is velvety, pink, and that of the large intestine is grey, folded, no nidal changes are revealed.

Reproductive organs have a usual structural pattern.

The microscopic study of organs of dogs of the experimental and control groups did not either reveal any differences between them.

Microscopic examination of structures of the studied organs of all animals which was conducted in 18 months after the material implantation demonstrates the same results.

Cerebrum neurons have a pyramidal or polygonal form, are surrounded by unstructured glial cells with a clear-cut nucleus.

Into the subcortical layer there are bundles of nerve fibers of different nature, aggregates of neurons in the form of solitary nuclei.

Loops of plethoric capillary in the anterior lobe of hypophysis are surrounded by secretory cells with principally oxyphilic cytoplasm.

The follicles of the medium lobe are of a rounded form, they are numerous and filled with an unstructured colloid. In the posterior lobe of neuropils there are some solitary follicles.

The salivary glands consist of intensively basophilic glandular cells, the saliva-excreting ducts are free, and the stroma is weakly developed.

Into the thyroid gland the stroma is weakly developed, the follicles have an oval form and are filled with an unstructured colloid.

Into the cortex part of thymus there are numerous aggregates of lymphocytes, the lymphocytes of the cerebral part are less numerous.

The lungs have well expanded alveoli, their cavities are free and alveolar walls are thin and filled with blood.

In the lumen of the large bronchial tubes there is insignificant amount of unstructured exudation.
The myocardium consists of bunches of cross-streaked fibers with safe eccentrically located nuclei.

The small glandular cells of the cortical layer of the adrenals are arranged layer-by-layer. Into the cerebral layer there are polygonal chromaffin cells and free sinuses.

In the kidneys there are glomerules of a rounded form, the subcapsular cavity is free, the structure of capillary loops is safe.
The excretory part of the nephron has a structure corresponding to its parts. The stroma is of a usual structure.

In the liver the stroma is weakly developed, the hepatic lobes are of a prismatic and oval form, the central veins are free, the hepatocytes have a precise structure, the sinusoid capillaries are plethoric.

In the pancreas equally basophilic excretory glandular cells of the acini, the epithelial pavement of the excretory ducts is not injured.
The insular apparatus is in a thin capsule, it contains light polymorphous cells of a usual structure.

The walls of the stomach and intestines have a usual structural pattern.

The spleen consists of large sinuses containing erythrocytes and lymphoid follicles of normal size.

In the lymph nodes there are polymorphous follicles consisting of small lymphoid cells and a weakly developed stroma.

2.3.2. Study of the implantation area (implantation test)

Roundish material aggregate enclosed by thin capsule is seen in the place of subcutaneous injection of the material into fatty tissue of the experimental group. The dimensions of the aggregate of the material after 18 months were in average 3x2x1.5 cm. Visually the material is not changed in comparison with the initial.

The morphological study of the tissue reaction at implantation zone showed that material keeps its structure and remains homogeneous. Round the bulk of the implantate there is formed a very thin capsule of connective tissue with a thickness from 50 to 150 microns); in the capsule small aggregates of large macrophages with foamy cytoplasm are observed. These cells ingest the material which remains in their cytoplasm in the form of vacuoles.
In places the capsule separates the bulk of the material from the areas which consist of a porous cellular tissue. An intensive resorption of the material by macrophages takes place in these areas. The bulk of the material remains homogeneous and is not infiltrated by cells and that accounts for its long-term resistance.
An insignificant lymphocytic and macrophage infiltration with solitary plasmatic cells is marked in the surrounding adipose cellular tissue and the muscular tissue.

2.3.3. Gonadotoxicity

The testicles of the dogs of both groups have a usual structural pattern. An epithelium of seminiferous tubules is active; there are all cells of spermatogenesis, many mitotic dividing cells and that speaks in favor of the reproductive function safety.

3. Conclusion on chronic experiment

The study showed that the “ARGIFORM” material is well tolerated by animals, does not affect their general status, behavior, weight, as well as the hematological indices and the functional status of the basic organs and systems of the animal organism, according to the indices of the used biochemical tests.

This all attests absence of toxic action of the “ARGIFORM” material.

Macroscopic and microscopic examinations of the internals of animals after subcutaneous implantation of the material did not either reveal any pathological changes connected with a toxic action of the material.

The histological study of testicles attests safety of the reproductive function.

The histological study of tissue reaction in the zone of implantation of the material to laboratory dogs points to the fact that the material is a bioinert one, does not cause any inflammatory reaction of tissue, is surrounded with a thin capsule of connective tissue and is not subjected to a noticeable resorption for a long period. The resorption of the material by macrophages was observed only in boundary layers.

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