TRANSLATED FROM RUSSIAN

SUBCHRONIC EXPERIMENT

1. The subchronic experiment included the following trials:

1.1. The subchronic toxicity of the material was researched according to GOST R ISO 10993.6 -99 "Medical goods. Studying of biological action of medical goods. Part 11. Study of general toxic action".

1.2. The local action of the material after the implantation (implantation test) was researched according to GOST R ISO 10993.6 -99 "Medical goods. Studying of biological action of medical goods. Part 6. Study of local action after implantation".

1.3. The gonadotoxic action of the material was studied in accordance with GOST R ISO 10993.10-99 "Medical goods. Studying of biological action of medical goods. Part 3. Study of genotoxicity, cancerogenity and toxic action on the reproductive function" by the condition of the histostructure of male reproductive organs".

1.4. The pathomorphological study of the internal organs of the experimental animals was done in accordance with GOST R ISO 10993.10-99 "Medical goods. Studying of biological action of medical goods. Part 3. Study of genotoxicity, cancerogenity and toxic action on the reproductive function".

The experimental animals were kept in accordance with GOST R ISO 10993.2-99 "Medical goods. Studying of biological action of medical goods. 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 cannulae is put into a separate branded carton box. The marking, description and the trademark are printed on the boxes.

2. Experiment description and results

2.1. Experiment description

The subchronic experiment was carried out on 40 nondescript male white rats with the body mass of 180-200 g. The material was introduced subcutaneously by means of a standard syringe. The animals were kept in full correspondence with the sanitary norms.

The experimental animals were divided into 2 groups:
- quartz glass plates were subcutaneously implanted to a control group (18 animals);
- A subcutaneous introduction of 0.8 ml of the the material which constitutes 4.0-4.4 ml for 1 kg of the body mass, was made by means of a large puncture cannulae to an experimental group (22 animals).

In order to evaluate the main factors characterizing the functional and the structural changes of internals and systems were used the following tests, listed below
(table #1).

Table #1
Investigated indices, methods and apparatus used in the subchronic experiment

Systems and functions	Methods of research	Apparatus
Integral indices	outward appearance, behavior, state of coetaneous integuments, hair and mucous membranes, food and water consumption	Visual observation
Liver functions	bromesulphalein test, activity of transaminases (ALT and AST), phosphatase (Shf), lactatdehydrogenase (LDG), cholynestherase (ChE), leucynaminopeptidase (LAP), total amount of protein in blood serum, protein in blood serum, total amount of cholesterol in blood serum, blood sugar, total and direct bilirubin	FP- 901 analyzer, automatic analyzer Technicon SMA16/60, refractometer IRF-22, electrophoresis apparatus with a phoregram analyzer AF-1. Photometer KFK-3
Functions of kidneys	urea nitrogen content, uric acid content, creatinin content, potassium and natrium content in blood serum	Spectrophotometer Shimadgzu UV-160A, Japan
Redox processes	content of sulphhydril (SH) groups and of malonic anhydride (MDA) in the blood serum	Spectrophotometer Shimadgzu UV-160A, Japan
Hematological indices of peripheral blood	Content of hemoglobin, red corpuscles, leucocytes, fibrillation period	Photometer KFK-3 (Russia), counter of formed elements of blood PICOSCALE PS-4, Medicor, Hungary
Pathomorphological research of the implantation area, internal organs, gonadotoxicity	Histological sections of organs and tissues in the implantation zone	Paraffin-embedded specimens fixed in neutral formalin; the sections were stained by gematoxilin-eosin, were seen in a photo microscope IMOVELL – CARL ZEIS YENA UTHV, Germany

2.2. Results of subchronic toxicity study.

Integral indices

During all the observation period there were neither deaths of the experimental animals, nor changes of their outward appearance, behaviour, motion activity in comparison with the control animal group.

Function of the liver

According to the data of the bromesulphalein test (table #2), the excretory function of the liver of the rats of the control group does not differ from that of the experimental group.
The activity of the ferments of ALT, AST, ChE, LDG, Shf and LAP, as well as the levels of total

protein, albumin, cholesterol, sugar, general and direct bilirubin in blood serum of both groups of rats do not differ statistically (tables #3 and 4) which testifies of the absence of hepatotoxic action of the material.

Kidneys function

The experimental animals had no statistically significant differences in the urea nitrogen content, uric acid content, creatinin content, potassium and natrium content in blood serum in comparison with the control animals (table #4) which testifies of the absence of nephrotoxic action of the material.

Redox processes

The study of a possible effect of the material on the level of peroxide lipid oxidation (PLO) was made with the help of evaluating of the content of nonprotein SH groups, malonic anhydride (MDA) in blood serum of animals.

The overwhelming part of the SH groups is constituted by reduced glutadion which play a significant role in detoxication processes. MDA is a secondary product of PLO and its level characterizes the intensity of the peroxide processes of an animal.
The content of SH groups was determined photocolorimetrically by an ultramicromethod, and the MDA level was determined by spectrophotometrical measuring of its reaction with c 2-thiobarbiturate acid.
The results shown in table #5 testify the absence of differences in the SH groups and MDA in a blood serum of the control and experimental animals.
Thus, the presence of the material in the animal for 2.5 months does not effect the peroxide lipid oxidation level.

Hematological indices

The indices of the content of hemoglobin, red count, the color index of the blood, thrombocytes and leukocytes content, hematocrit and blood clotting time of the experimental and the control animals fluctuate within a physiological standard; however, there are no statistically significant differences between the groups (table #5).
This is an evidence of absence of toxic effect of the material on the structure and the functional status of peripheric blood of the experimental animals.
The results of the hematological and biochemical inspection of the rats are shown in tables #2-6 as M±m where M is the arithmetic average of the measurement results, m is the standard deviation of the measurement results.

Determination of the relative masses of the internals

Determination of the mass of the internals of the animals was made after morrow of the experiment.

The results of the determination of the weight coefficients of the internals showed that there were no deviations in the weight coefficients of the masses of liver, spleen, kidneys, thymus, and testicles of the experimental group in comparison with the control group.
The results of the determination of the weight coefficients of the internals are shown in table #7, also as M±m.

Table #2
Bromsulfalein test, mg%

Animal groups	Bromsulfalein concentration in plasma in 2 min.	R
Control	10.73 ± 0.37	>0.05
Test	10.08 ± 0.28	>0.05

Table #3
Enzymological indices of blood serum

Indices	Control	Experiment	R
Alanine aminotransferase, units/l	36.29 ± 1.36	35.07 ± 1.23	>0.05
Asparagine aminotransferase, units/l	116.73 ± 4.26	119.6 ± 3.86	>0.05
Lactatdehydrogenase, units/l	2077.88 ± 46.91	2172.50 ±4 9.10	>0.05
Cholinesterase, units/l	151.28 ± 5.52	143.15 ± 3.80	>0.05
Alkaline phosphatase, units/l	42.06 ± 1.42	40.88 ± 1.35	>0.05
Leucinaminopeptidase, units/l	30.06 ± 0.51	29.22 ± 0.62	>0.05

Table #4
Biochemical indices of blood serum

Indices	Control	Experiment	R
Total amount of protein, g%	6.84 ± 0.30	7.03 ± 0.32	>0.05
Albumins, g%	2.89 ± 0.10	2.66 ± 0.08	>0.05
Cholesterol, mg%	70.98 ± 2.56	70.70 ±3.78	>0.05
Sugar, mg%	113.67 ± 3.64	114.74 ± 5.4l	>0.05
Urea nitrogen, mg%	30.13 ± 1.14	27.38 ± 0.87	>0.05
Uric acid, mg%	3.65 ± 0.08	3.43 ± 0.09	>0.05
Natrium, mequ/l	138.00 ± 3.97	138.11 ± 3.53	>0.05
Potassium, mequ/l	7.44 ± 0.19	7.19 ± 0.19	>0.05
Acid gas, mequ/l	18.44 ± 0.70	19.33 ± 0.54	>0.05
Carbon dioxide, mg%	0.72 ± 0.01	0.75 ± 0.01	>0.05
Total bilirubin, mg%	0.27 ± 0.01	0.25 ± 0.01	>0.05
Direct bilirubin, mg%	0.12 ± 0.06	0.14 ± 0.01	>0.05

Table #5
The level of nonprotein SH groups, mmole/ml and MDA, mmole/ml in the blood serum

Animal groups	Nonprotein SH groups	MDA	R
Control	0.30 ± 0. 01	69.62 ± 1.98	>0.05
Test	0.30 ± 0.01	71.92 ± 2.13	>0.05

Table #6
Hematological indices

Indices	Control	Experiment	R
Hemoglobin, g%	12.74 ± 0.11	12.93 ± 0.05	>0.05
Red corpuscles, m/mm	5.96 ± 0.15	6.30 ± 0.20	>0.05
Thrombocytes, ths/mm	812.20 ± 21.98	771.43 ± 15.15	>0.05
Leukocytes, ths/mm	9.35 ± 0.28	9.93 ± 0.33	>0.05
Hematocrit, %	41.49 ± 1.25	45.00 ± 1.3	>0.05
Clotting time, sec.	39.10 ± 1.38	39.54 ± 1.23	>0.05

Table #7
Weight coefficients of rats' internals

Internals, mg	Control	Experiment	R
Thymus	2.28 ± 0.03	2.20 ± 0.04	>0.05
Spleen	2.85 ± 0.12	2.82 ± 0.08	>0.05
Kidneys	8.27 ± 0.28	8.90 ± 0.22	>0.05
Testicles	10.44 ± 0.31	11.30 ± 0.35	>0.05
Liver	51.43 ± 1.36	50.72 ± 1.25	>0.05

2.3. Pathomorphological research

2.3.1. Methods, description and results

The pathomorphological research of the tissue in the material implantation area was conducted after 7, 14 and 75 days; the histological research of the internals (liver, kidneys, spleen and testicles) was carried out on the morrow of the experiment.

Tissue samples were fixed in non-inhibitive formalin and embedded in paraffin; the sections were stained by gematoxilin-eosin, pyrofuchsin, according to Van Gizon, and with toluidine blue for acidic glycosaminglycanes (GAG).

2.3.2. Implantation test

In the control group the glass plate was implanted in 7 days round the implant macrophage and lymphocyte reactions of the tissue with an admixture of neutrophils are observed which is evidence of a moderate aseptic inflammation as well as of proliferation of fibroblasts.

In 14 days a still immature capsule of connective tissue forms round the implant. In 2.5 months a not very thick mature fibrous capsule of connective tissue was found round the implant. There are a few large macrophages and isolated gigantic multinuclear cells in the capsule's boundary zone near the implant. There are small lymphomacrophagal infiltrates with solitary neutrophils in the capsule and in the adipose cellular tissue surrounding it.
In the experimental group the research of the tissues of the implantation zone of the material in 7, 14 and 75 days after its subcutaneous introduction gave the following results.

In 7 days the material is compact, has a cellular structure and is eosinophilically tinged. In places there are small solitary aggregates of large macrophages. There is no neutrophilic infiltration of the material. A very thin porous capsule of connective tissue only begins to form round the material.

There are practically no neutrophils in it and the cellular tissue surrounding it and the tissue has a minimal edema. The macrophagal reaction is moderate; the proliferation of fibroblasts is very weak. In the boundary layer between the capsule and the material there are solitary macrophages and practically no giant cells. Outside the capsule there are small aggregates of the material which has separated from the compact bulk. These aggregates are subject to the resorption of large macrophages with foamy cytoplasms.

In 14 days the bulk of the material remains compact and, as before, has a cellular structure. There are solitary cells (macrophages) in it. The capsule of connective tissue is immature and extremely thin. The fibroblastic reaction is as weak as before. The quantity of the macrophages of the boundary layer increases, and they form a continuous row, and there are also solitary giant cells. In places there are small fragments of gel surrounded by macrophages and more seldom by giant cells. The majority of these fragments are resorbed and in their place there are empty cells. The resorption of the bulk of the implant takes place only in small areas on the periphery where in the material there is proliferation of fibroblasts and macrophages. There is practically no inflammatory infiltration.

In 2.5 months after the implantation the structure of the material is slightly changed. Small-celled and small-grained areas appear in the material. The capsule of connective tissue acquires a more fibrotic structure but remains very thin. The quantity of macrophages in the boundary zone decreases significantly, there is no inflammatory infiltration. In places the capsule is thickened because of the remains of the material or of the cellular tissue after the resorption of the material. In the thickened parts there are macrophages and solitary giant cells.

2.3.3. Research of the internal organs

The internal organs (liver, kidneys, spleen and testicles) were researched after the experiment was finished.
There were no significant differences in the histological structures of the control and the experimental groups.

In the liver, both in the experiment and in the control, there was a moderate plethora of central veins, branches of the portal, interbeam capillaries. The structure of the lobes and beams is not broken, the quantity of Kupffer's cells is usual; a moderate lymphohistiocytic infiltration in the stroma round the triads is registered both in the experiment and in the control. An insignificant proteinosis of hepatocytes is equally expressed in the control and in the experiment.

In the kidneys the histostructure of the organs remains the same. The cellular compositions of the glomerules and the stroma of kidneys the control are usual and do not differ from those of the experiment. The epithelium of convoluted and straight tubules is, in general, not changed; in places a certain granularity of cytoplasm is registered but it is equally expressed in the control and in the experiment.
In the spleen of part of the animals plethora of the red pulp is noted, equally expressed in the control and in the experiment. The lymph follicles are of the usual size and have embryonic centres. In the marginal zones of the follicles there is a moderate quantity of plasma cells, the macrophagal reaction is the same.

2.3.4. Gonadotoxicity

In the testicles the histostructure of the organ remains the same. No dystrophic or necrotic changes were registered. The spermatogenous epithelium of tubules is active, all the typical cellular

associations are displayed: spermatogonia, spermatids, primary and secondary spermatocytes. There are many cells with an active fission. All these are evidences of the fact that the reproductive function is preserved.

3. Conclusion on subchronic experiment

The conducted subchronic research of the ARGIFORM material shows that in the experiment on rats a long-term staying of the material in the animal does not cause any statistically reliable changes in comparison with the control, according to the main biochemical indices which characterize the functions of the liver, kidneys, redox processes, as well as according to the hematological indices. That attests absence of toxic action of the material which is also confirmed by a pathomorphological research which has not display any changes of the histostructure of the internals.

The histological research of the testicles points to the preserved reproductive function of the animals.

The histological research of the implantation area of the rats shows a very weakly expressed tissular reaction to the implantation of the material and a high degree of its biocompatibility. The aseptic reaction of the tissue is very weak on the first days after the implantation and is practically absent in 2 weeks and и 2.5 months. The capsule forming round the implant is very thin since the material causes a very weak fibroblastic reaction of the tissue.

The Head of laboratory experimental patomorphology
Moscow Medicine Academy of I.M.Setchenov,
MD, the professor _*Signature*_ A.B.Shehter