TEST PROTOCOL No. III 5 - 2008

IMPLANTATION TEST

Cytological and histological examination of the synovial fluid from the synovial membrane and cartilage of the hock joints of rabbits after administration of the advanced gel product.

Local effect of the materials after the implantation (implantation test) was examined according to GOST R ISO ISO 10993- 1999 Biological evaluation of medical devices, part 6 – Tests for local effects after implantation.

For the duration of the experiment, the animals were kept according to GOST R ISO 10993-1999, Biological evaluation of medical devices, part 2 – Animal welfare requirements.

The material was provided in the sterile condition, in disposable syringes, blister-packed and further packed in individual paper boxes.

Methodology

The experiment was conducted on 12 chinchilla rabbits. 1 ml of gel was injected into the articular cavity of a rabbit's right hock joint. 1 ml of saline was injected into the articular cavity of the rabbit's left hock joint, for control purposes. The rabbits were withdrawn from the experiment on day 1, 3, 7, 14, 30, 90 after the injection. The joint as opened, tissues from the synovial membrane and the articular cartilage were taken for histological and histochemical analysis, they were fixed in neutral 10% formaldehyde solution and embedded in paraffin. 4-5 micron sections were stained with hematoxylin and eosin, picro-fuchsin solution acc. to Van-Gieson, and with toluidine blue for acidic glycosaminoglycans. 144 histological specimens were examined altogether.

Smears of the articular cavity contents (gel and synovial fluid) were taken for cytological analysis. The smears were fixed in 96° alcohol, Giemsa stained, examined at XI000 magnification, and the percentage of different type cells was calculated. Altogether 40 smears were examined.

1. Macroscopic examination.

After opening control joints (administration of saline) there is a small amount of transparent synovial fluid of moderate viscosity. The synovial membrane consists mainly of fatty tissue. Articular cartilage is thin, whitish, with a smooth glossy surface.

24 hours after administering the gel, the cavity of the joints under examination contains a transparent substance with the gel and the synovial fluid forming a unified substrate. The viscosity of this substance is higher than that of synovial fluid and is closer to that of the intact gel.

The synovial membrane is not hyperaemic, it apparently maintains its normal structure, and cartilaginous plate also remains the same.

72 hours after administration the articular cavity also contains the viscous transparent substance. Because there is no opacity of the substance, as well as hyperemia

or edema of the synovial membrane, it means that there is no apparent inflammatory reaction.

7-14 days after the administration the concentration of the gel in the articular cavity drops, as well as viscosity. Synovial membrane and the cartilage continue to show no signs of pathological changes.

2. Histological and histochemical analysis of synovial membrane and articular cartilage.

24 hours after the injection.

Rabbit No. 5.

In the left joint **(control)** the synovial membrane is of fatty (adipose) structure. It consists mainly of adipose tissue with a moderate amount of capillaries. Some internal areas of the membrane contain an elevated amount of connective tissue in the form of loose collagen fibers, which are Van Gieson's stained red, and fibroblasts. The internal surface of the membrane is lined with one layer of synoviocyte. In some areas it is a continuous layer, in other it interrupts. There are small areas where synoviocytes form two layers of cells. After toluidine blue staining the cytoplasm of these cells results in metachromasy, which means that they contain acidic glycosaminoglycans, which these cells secret into the synovial fluid.

Synoviocytes are relatively large cells with round and elongated nucleus and moderately apparent cytoplasm. There are two types of cellular elements among them: macrophage-derived A-cells and B-cells of fibroblast origin. The subsynovial membrane consists mainly of lipocytes and also of fibroblasts, some macrophages, and lymphocytes.

The articular cartilage of the same joint contains the following structure: the internal surface of the articular plate is covered with the cell-free, transparent and thin strip. Deeper, lies the superficial, intermediary and the deep zones of the cartilaginous plate, which consist of numerous chondrocytes, each of which is formed by round nucleus and the cytoplasm rim and is surrounded by light lacuna. Between the cells, there is the extracellular matrix consisting of thin collagen fibers, Van Gieson's stained fuchsinophil, and of proteoglycans (aggregates), which are revealed with metachromasy, with toluidine blue staining. Proteoglycans are evenly distributed. Collagen fibers are not revealed at light microscopy because they are masked with proteoglycans and for this reason, the matrix seems homogeneous, however, the Van-Gieson fuchsinophily is well visible and uniform.

The deep layer of the cartilaginous plate is of the same structure as the intermediary, and the chondrocytes form vertical columns. Occasionally lime salt deposits may be found in the deep layer.

The synovial membrane of the right joint **(experiment)**, in which the gel was administered, has the same structure as that of the control joint. The main elements visible here are fatty tissue villi and small fibrous areas. The difference from the control joint in a cellular composition is minimal. The synoviocytes lie in a single or, in some areas, double layer. The synoviocyte structure is normal, gel deposits on the surface are not found, acidic

glycosaminoglycans concentration in them is the same as in the control material. A total number of lymphocytes does not grow compared to the norm, and the concentration of macrophages in the superficial layer is a little higher. There are several small areas wherein the subsynovial layer, there are separate neutrophilic leukocytes. Capillaries are mildly filled with blood, no tissue edema.

Cartilaginous plate in this joint is not in any way different from the control joint. There are no dystrophic changes of chondrocytes in any zones of the cartilage. The distribution and the intensity of collagen and proteoglycan coloring are in the normal range. The structure and the architectonics of chondrocytes are normal at all times.

Rabbit No. 6.

The synovial membrane in the left control joint is complete of a fatty structure. Synoviocytes lie in a single layer almost all over, except for two small areas where the layer is double. Blood vessels are few, moderately filled with blood, lymphocytes and macrophages are few.

The cartilaginous plate's structure is normal, in no way different from the cartilage of the control joint of rabbit No. 5. The internal glossy plate is plain, chondrocytes' structure is standard, and the extracellular matrix is rich in proteoglycans and well stained with Van Gieson's solution.

The synovial membrane of the right experiment joint has a fatty tissue structure; for the most part, it is covered with a single (or partly double) layer of synoviocytes. However, unlike in the control articulation, here there are several small areas of synoviocyte proliferation (mainly A-cells), which form 4-5 cell layers. However, no gel deposits were found in these areas. Acidic glycosaminoglycans concentration is normal.

Capillaries are a somewhat more blood filled, and the number of macrophages in the subsynovial layer is a little higher. The growth of the concentration of macrophage A-cells in the synoviocyte layer means that the phagocytic function of these cells (gel phagocytosis). No tissue edema is present.

No dystrophic changes appear in the cartilaginous plate, and the matrix's histochemical analysis shows standard results. No differences from the control articulation are found.

3 days after injection.

Rabbit No. 7.

In the synovial membrane of the left control articulation, there are parts of the fatty structure and vascular (areolar) structure. Synoviocytes are positioned in a single or double layer in the fatty tissue structure areas, and the membrane tissue has a moderate amount of lymphocytes and macrophages and some blood vessels. However, there are small parts where the lymphocyte and macrophage concentration is somewhat higher.

Areolar membrane parts contain much more blood vessels, more lymphocytes and macrophages, and some internal surface areas contain the moderate proliferation of synoviocytes, with three-four layers.

Certain differences in the cellular element concentration between the control articulation of rabbit No. 7 and articulations of the above-mentioned rabbits (No. 5 and No. 6) may be due to the individual characteristics of the animal.

Cartilaginous plate in this articulation is of normal structure, without dystrophic changes of chondrocytes, and the extracellular matrix contains the normal amount of proteoglycans and collagen.

The synovial membrane of the right experiment articulation has a fatty structure, synoviocytes lie in a single or a double layer here. Only one small area demonstrates some low proliferation (not more than in the control articulation) of synoviocytes, mainly macrophage type A. Adipose tissue of the membrane contains some nidi with a higher concentration of macrophages and lymphocytes, but they are of the same nature as in the control articulation.

The cartilaginous plate of the same articulation demonstrates no changes compared to the control articulation. Dystrophic changes are not found, the matrix if structurally and histochemically standard.

Rabbit No. 8.

The synovial membrane in the left control articulation is of fatty tissue, the synoviocytes lie in a single or a double layer. The concentration of the lymphocytes and the macrophages is negligible. The blood vessels contain a low amount of blood.

The cartilaginous plate of this articulation is of standard structure, no dystrophic changes, the matrix is not changed.

The synovial membrane of the right experiment articulation is of fatty type. For the most part, it is covered with a single layer of synoviocytes, but in one of the parts there is a synoviocyte proliferation and a multi-layer is formed. This would contain mostly macrophage A-cells with enlarged cytoplasm, which is an indicator of phagocytosis activation. Blood vessels of the membrane tissues are moderately filled with blood, macrophages are more numerous and no neutrophils are found.

The cartilaginous plate of this articulation has a normal structure and is no different from the cartilaginous plate in the control articulation.

1 week after injection.

Rabbit No. 3.

The synovial membrane of the left control articulation is of fatty tissue structure, the synoviocytes lie in a single layer, a double layer only in some small parts. Blood vessels are few and full-blooded; a number of lymphocytes and macrophages is within normal range.

The cartilaginous plate in the same articulation has normal structure. Chondrocytes show no signs of dystrophy; the matrix is rich in proteoglycans.

The synovial membrane of the right experiment articulation also has a fatty structure, synoviocytes lie in a single or a double layer here. Small aggregates of the mini-vacuolated gel can be found on the surface of the synovial membrane and in two internal parts. Synoviocyte proliferation in these areas is not found. No inflammatory infiltration found around the gel as well. Occasionally, around and inside of the described gel aggregates macrophages resorbing the gel can be seen in the membrane.

The structure is fully maintained in the cartilaginous plate of this articulation, no gel deposits found. No dystrophy changes in the cells, the matrix is of standard structure.

Rabbit No. 4.

The synovial membrane of the left control articulation is of fatty tissue structure, one fragment is classified as fibrous, and the synoviocyte layer is mostly a single layer. A total number of the lymphocytes and macrophages is negligible and they are evenly distributed across the membrane tissue. Blood vessels are few.

The cartilaginous plate of this articulation is of standard structure, no dystrophic changes, the matrix is not changed.

The synovial membrane of the right experiment articulation is of fatty tissue structure, the synoviocytes are mostly arranged in a single layer, but there is a small region where, due to moderate proliferation, they form several layers. The blood vessels contain a large amount of blood.

Same as with rabbit No. 3: several small gel aggregates without inflammation around can be found in the membrane tissue. Part of the gel is actively resorbed by macrophages.

The cartilaginous plate of this articulation is of standard structure, no dystrophic changes; the matrix is not different from the controlled tissues.

2 weeks after injection.

Rabbit No. 1.

The synovial membrane in the left control articulation is of fatty tissue, the synoviocytes are arranged in a single or in some areas a double layer. Macrophage concentration is low, blood vessels are few.

The cartilaginous plate of the same articulation shows no deviations from the normal level: no dystrophic changes of chondrocytes, the matrix are also the same.

The synovial membrane of the right experiment articulation is of fatty type. The Larger surface area is covered with a single or a double layer of synoviocytes, just a few proliferation areas, also there are some proliferation areas where synoviocytes are arranged in a multilayer. Gel deposits inside and on the surface of the membrane are not found.

The cartilaginous plate in this articulation is the same as that of the control articulation if we compare the structure and histochemical analysis.

Rabbit No. 2.

The synovial membrane in the left control joint is a fatty structure. Synoviocytes are arranged in a single or a double layer. Blood vessels are a few; lymphocyte and macrophage concentration is low.

The cartilaginous plate in this articulation has normal structure, no peculiar features.

The right experiment articulation also has a fatty synovial membrane. Synoviocytes are arranged in a single layer, no synoviocyte proliferation is found. A tiny gel fragment was found in a fatty tissue area, surrounded by macrophages; apparently, this was a piece of the remaining gel almost completely resorbed by this point.

The cartilaginous plate in this articulation is the same as that of the control articulation if we compare the structure and histochemical analysis. No dystrophic changes.

1 month after injection.

Rabbit No. 9.

The synovial membrane in the left control articulation is of fatty tissue in some places and fibrous in other places. The blood vessels contain a moderate amount of blood. Synoviocytes arrange in a single layer, partially double layer in the fatty lining, the lymphohistiocytic infiltration is minimal, mainly around the blood vessels.

The cartilaginous plate in the same articulation has normal structure. The superficial layer contains small-sized chondrocytes; the middle deep layer contains large chondrocytes with clearly distinguished lacunae, no dystrophic changes to the cells. The cartilaginous matrix contains a lot of proteoglycans; the structure remains absolutely the same.

The right experiment articulation has a fatty synovial membrane. Synoviocytes are arranged in a single layer, but in some areas, they form a double or triple layer. No proliferation nidi are found. Perivascular lymphatic and histiocytic infiltration is not considered at the control level.

The cartilaginous plate in this articulation is of normal structure, chondrocytes have clearly distinguished lacuna, round nucleus, and unchanged cytoplasm. The matrix of cartilaginous tissue remains the same.

Rabbit No. 10.

The synovial membrane in the left control articulation is of fatty type, synoviocytes arranged in a single layer, blood vessels moderately filled with blood, few lymphocytes and histiocytes in the perivascular space.

The cartilaginous plate is not modified, its surface is smooth, and chondrocytes demonstrate signs of dystrophic changes, the matrix of standard structure.

The first experiment articulation has a fatty type synovial membrane, synoviocytes arranged in a single layer, no gel deposits found, blood vessels moderately filled with blood, lymphohistiocytic reaction at the control level.

Cartilaginous plate in of this articulation has normal structure; chondrocytes are distributed evenly and demonstrate no dystrophic changes. Cartilage matrix is rich in proteoglycans, no lytic lesions found in it.

3 months after injection.

Rabbit No. 11.

The synovial membrane in the left control articulation is of fatty type, synoviocytes arranged in a single layer. In some small areas the synoviocyte layer is double, blood vessels are filled with blood, and some lymphohistiocytic cells are found in the perivascular spaces.

The cartilaginous plate of this articulation has normal structure; chondrocytes are large cells with lacunae, no changes in the matrix.

The synovial membrane of the right experiment articulation is fatty and fibrous. Most of its surface is lined with a single layer of synoviocytes; however, there are small areas where the layer is thicker, with synoviocyte proliferation. Small fragments of the resorbed gel can be seen in these areas. Occasionally they are surrounded by multinucleated giant cells, like foreign body giant cells. No gel found in the synovial membrane tissue, inflammatory infiltration not found. Blood vessels are moderately filled with blood.

The cartilaginous plate has normal structure, cells without changes, no structural changes in the matrix, proteoglycan concentration the same as in the control material.

Rabbit No. 12.

The synovial membrane of the left control articulation is fatty and fibrous. Synoviocytes arranged in a single layer, blood vessels moderately filled with blood, no inflammatory infiltration.

The cartilaginous plate has normal structure, no changes in chondrocytes and matrix found.

The first experiment articulation has fatty and fibrous synovial membrane, synoviocytes arranged in a single layer almost across the whole of the surface. A small area of gel deposit surrounded by macrophages and giant cells found in the subsynovial membrane.

The cartilaginous plate has no changes; chondrocytes and matrix maintain the same structure and proteoglycan concentration as in control material.

3. Cytological examination of the contents of articular cavity

Hyaluronic acid in the fine granular form was found in the synovial fluid of the control joints. The percentage of cellular composition of the synovial fluid did not very much for animals withdrawn from the experiment at different times (see table 1).

Desquamated synoviocytes – relatively large cells with round nucleus and well-distinguished cytoplasm rim without signs of phagocytosis – were a dominant cellular element. On average they were 91.1% of all the cells. Synoviocytes with the signs of phagocytosis (vacuolated or granular cytoplasm) were few (0.9%). Monocyte-macrophages of hematogenous origin were also present (2.9%), and they were smaller in size and had a bean-shaped nucleus. The rest of the cells were lymphocytes (4.5%) and neutrophils (0.6%).

The gel in the experiment articulations maintained cytological homogeneity 24 hours after injection, but after 3 days vacuolization and fibrillization started in it, which are signs of cell-free lysis. By the 7th day this process became more active, and by the 14th day, the amount of gel is very little. 1-3 months after the injection only occasional small remaining pieces of the gel can be rarely found in the synovial membrane. The gel is resorbed mostly by synoviocytes.

Normally synoviocytes (lining cells of the synovial membrane) consists of two populations: macrophage-derived A-cells and B-cells of fibroblast origin. Based on the cytology data (see table), the phagocytosis of the gel starts on the first day, runs faster on days 3-7 and is mainly performed by A-cells migrating to the articular cavity, and to a much lesser degree by hematogenous macrophages (from blood monocytes). Phagocytizing synoviocytes are large; their cytoplasm is foam-like or, less often, granular.

Phagocytizing cells, which are only 0.9% of cells in intact articulations, become over 50% already on the first day after the gel injection, and 73% of all the cells on the 3rd day. On the 14th day their proportion drops to 50%, but even after 3 months they still make up around 19.2% of the cells. At all-time marks phagocytizing synoviocytes are much more numerous than phagocytizing macrophages of hematogenous origin; the latter also are smaller and have the bean-shaped nucleus.

The percentage of neutrophilic leukocytes in a smear, which is normally 0.6%, grows to 3.5 - 3.1% on the 1-3rd day after the gel injection. This indicates that the aseptic inflammation reaction to gel injection is very mild in the synovial membrane and is quickly neutralized: after 7 days neutrophils are 2.2% and after 14 days 1.1%, on the 90th day 0.7%, i.e. the percentage grows to normal. Lymphocyte percentage remains almost the same. Percentage of non-phagocytizing synoviocytes drops considerably compared to control material on day 1-3 but then grown gradually to day 14-90.

Percentage of cells in synovial articular fluid smears.

Table 1

% of cells	control	experiment					
		1 day	3 days	7 days	14 days	1 month	3 month
1. Non-phagocytizing synoviocytes	80.6	26.0	12.4	40.9	46.8	59.6	64.3
2. Monocyte-macrophages	2.9	8.4	3.1	3.0	3.4	3.2	3.1

3. Phagocytizing cells -3a. Synoviocytes 3b. Macrophages	0.9 0.8 0.1	58.2 38.2 20.0	73.3 74.2 18.8	50.1 34.2 15.9	44.5 29.0 15.5	32.1 19.4 12.7	21.5 13.5 8.0
4. Lymphocytes	3.6	3.9	4.1	3.8	4.2	4.3	4.1
5. Neutrophils	0.6	3.5	3.1	2.2	1.1	0.8	0.7
Number of cells in the field of vision	2.7	19.4	21.5	9.6	6.4	6.2	5.1

Conclusion.

Cytological, histological and histochemical analysis of the synovial fluid, synovial membrane and cartilaginous plate of the rabbits' hock joints at different time marks after the injection of polyacrylamide gel containing silver in the articular joint has shown that the changes in the examined environments and tissues are minimal and disappear quickly. The macroscopic examination of the synovial fluid of the experiment articulations with the gel injected has shown that on day 1-3 the fluid was more viscous compared to control articulations, where the equal volume of saline was injected. The viscosity was higher due to synovial fluid and gel forming a conglomerate, as they unite in a uniform substrate that is not divided into components. This substrate remains clear, and as there is no opacity, it means that there is no inflammation in the joint.

The amount of gel drops 24 hours after the injection and even more after 14 days, due to which the substance loses viscosity, but the substance still remains transparent. 1 month after the injection the amount of gel decreases even further, and by the 3rd month, there is almost no gel in the articular cavity. Smear cytology has shown that 24 hours after the gel injection it remains homogeneous. By the 3rd day vacuolization and fibrillization start, which become more apparent by the 7th day. By the 14th day the percentage of gel drops considerably, and by month 1-3 the gel may not be found.

The decrease of the gel percentage happens both due to cell-free lysis and resobrtion by its macrophages. The main role belongs to macrophage synoviocytes (A-cells), which are desquamated from the synovial membrane surface. These cells amount to 70% of all the cells by the 3rd day (0.9% in control material). Percentage of neutrophilic leukocytes grows a little by day 1-3 (not more than 3%), which indicates that the aseptic inflammatory reaction to the gel injection is very weak. By day 14 a number of neutrophils drop to 1% of all cells. By this time the amount of phagocytizing synoviocytes and macrophages drops as well. 1-3 months after the injection a number of neutrophils are the same as in control material. The percentage of phagocytizing cells drops to 32% by month 1 and to 21% by month 3. This entire means that the gel is gradually removed (resorbed) from the articular lumen.

The synovial membrane of the control joints of all the animals' demonstrated fatty (adipose) and to the lesser degree fibrous structure. The surface of the membrane is covered with a single (or partially double) layer of synoviocytes secreting hyaluronic acid, which is revealed histochemically through

metachromasy with toluidine blue staining. Blood vessels of the membrane are few and are moderately filled with blood. Apart from fatty cells (lipocytes) the membrane contains a few lymphocytes and macrophages, evenly distributed.

Areas of areolar structure (with a higher number of blood vessels) were found in the synovial membrane in the control joint of only one rabbit (No. 7), and a number of lymphocytes and macrophages in these areas was higher as well. This is expected to be due to the individual characteristics of the animal.

Experiment joints demonstrate no edema and no vascular reaction, typical for synovitis, on day 1-3 after the gel injection. Neutrophilic infiltration is minimal, focal and is only seen under the layer of synoviocytes, where also the number of macrophages grows a little. The synoviocyte cover itself is a single layer, but in one animal on day 1 and in both animals on day 3 small synoviocyte proliferation nidi were found, mainly due to A-cell macrophages, which participate in the gel phagocytosis. In general, however, no inflammation has been registered. No difference in histology or histochemical composition between the animals withdrawn from the experiment on day 1 and day 3 was found.

By the 7th day after the gel injection, the synovial membrane still shows no signs of edema or inflammation. Synoviocyte proliferation areas were found only in one of the two animals, and they are not larger than those registered on the 3rd day time mark.

It should be mentioned that in both animals small aggregates of finely vacuolized gel were found under the synoviocyte layer in the membrane tissue. Inside the gel and around its fragments macrophages phagocytizing the gel can be seen. However, there is no inflammatory infiltration around the gel fragments, which indicates the gel is bioinert.

14 days after the injection one small proliferation areas was found only in one animal, and the other had a very small gel fragment found by macrophages and actively resorbed by them. 1 month after the injection there were no signs of any changes in the synovial membrane of both animals, 3 months later small fragments of gel were still present in the covering layer, which stipulated focal synoviocyte proliferation in the two small areas of synovial lining.

The articular cartilage in the experiment articulations maintains absolutely the same structure and histochemical composition as the control joints cartilage, at all-time marks. The cartilaginous plate of all the examined joints has normal structure, with no dystrophic changes of chondrocytes or extracellular matrix destruction, proteoglycan, and collagen concentration in the matrix is within the normal range. As the nutrition of non-vascular cartilaginous tissue in the articulation is provided by the diffusion of nutrient substances from the synovial fluid, the absence of the changes in cartilaginous plate means that the administration of the gel in the articular cavity has no effect on the cartilage metabolism.

Thus, the histological and the histochemical analysis of the synovial membrane of the articular cartilage show that there are no inflammatory changes (synovitis), not dystrophic or necrotic changes in the membrane and the cartilaginous tissue, which means that the gel is bioinert when injected into the articular cavity. Small synoviocyte proliferation areas registered at different times after the gel injection and small areas of gel incorporation into the synovial membrane have no effect on the state of the latter and do not cause synovitis. The cytological examination of the synovial fluid shows that

the gel is gradually resorbed in the articular cavity by the macrophage-derived synoviocytes (A-cells), which migrate from the synovial membrane, and, to a lesser degree, by hematogenous macrophages. The gel and the synovial fluid form complex compounds, which do not affect the metabolism of the articular tissues.

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