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STUDY ON BIOCOMPATIBILITY OF CHEMICALLY COATED TITANIUM WITH BIOLOGICAL HYDROXYAPATIT

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The reconstruction of large bone defects after traumatisms or after extirpation of tumoral formations, benefits today of surgical methods which morphologically and functionally remake of the bone tissue, using transplants or implants based on calcium phosphate. A remarkable characteristic of the phosphatic ceramic which differentiates biologically inert implants, is represented by its capacity to interact directly with bone tissue. The main objective of the study has pursued obtaining a biomaterial made of titanium with higher mechanical, physical and chemical properties with direct applications in oral and maxillofacial surgery. The study tries to emphasize the bioactive potential of the biomaterial obtained by coating titanium with biological hydroxyapatite as consequence of this immersion in blood plasma similar solution.

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1. Introduction

One part of the population suffers of severe diseases which consists of making sick or destroy the hard bone tissues and the costs due to the complicate surgical operations for the their solving are significant. At present, all over the world, a good part of the operations of reconstruction consists of the replacement of sick or destroyed tissues with metal implants (stainless steel, titanium or its alloys) or ceramic, based on aluminum oxide or zirconium oxide, to take over their mechanical functions. These are intolerable or biologically inert materials and, in time, it is forming a fibrous tissue characteristic to the reaction to the foreign body is taking place at their interface with the bone receptor bed. Thus, a layer is developed and within 10-15 years a displacement of the implant is taking place needing its replacement by a new surgery (1).

In the last decades, both as consequence of increasing the life expectancy and of the number of accidents of circulation or labor accidents, the need of materials for the repair of hard living tissues (bones and teeth) is higher and higher.

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It is well known that as we advance in age, a progressive deterioration of the mechanical resistance and of the bone density is taking place and from these reasons, the most vulnerable to fractures are the elder persons.

The most efficient material used is the biological material collected from the patient (self-graft) or from another person (allograft). However, the graft shows also a series of inconvenients (2): the person from which collecting is made, is submitted to new sufferance, because the peril of infection occurs and the quantity of bone that may be collected is limited (especially in case of children)(1). Another applied solution is the use of the preserved bone. This is very expensive as consequence of the expenses that must be made for the preservation investigations and for HIV and hepatitis detection (however these investigations guarantee no 100% safety). These are the reasons for which the attention of the specialists has been directed towards the achievement of synthetic materials which offer bones grafts. They must be accepted by the body without irritation or necrosis of the adjacent tissues, they must not produce genetic mutations and they must take over the mechanical and biological functions of the replaced bone (3.). The best results have been obtained with a series of materials generically denominated "bioceramics", used more and more frequently for the repair, reconstruction and replacement of hard, sick or destroyed tissues (4).

Given the requirements of the dental implant – the high mechanical resistance, the resistance to the corrosion of buccal liquid, biocompatibility with the surrounding tissue, it has been established that a very good solution is to coat the biologically tolerated titanium metal with a bioactive hydroxyapatite layer.

2. Material and method

Coating technology of the implant is established at the laboratory level by coating with a hydroxyapatite layer from a similar solution with the mineral part of the blood plasma. After establishing the layer coating technology, samples have been achieved in view of determining the biological characteristics of the coated layer.

In view of performing in vitro investigations 3 batches of models have been achieved. The first batch was Ti_6Al_4V titanium alloy that was shaped in small round disks with 3 mm diameter (marked T1). The second batch has been Ti6Al2NbTa alloy shaped in square parallelepipeds 5 mm lateral size and 3mm height (marked T2), and the third batch has been $Ti_{15}Mo_5Zr_3Al$ alloy shaped in disks with 5mm diameter and 2mm thick, cut in cylinders with 4mm diameter and height 10 mm (marked T3). Then the samples have been cleaned with acetone, ethyl alcohol and distilled water after which their surface is attacked with (4M-NaOH) at 60^{o}C for a period of 48 hours that leads to the occurrence on the surface of the samples of a sodium titanate layer. The samples are then washed with distilled water and dried at 40 ^{c}o during 24 hours after which they are submitted to a thermal treatment at 600^{o}C in an electric oven with programmer as follows: an increase of temperature from 0 ^{o}C to 600^{o}C with 300 ^{o}C/h, plateau at 600^{o}C during 1 hour and free cooling until 30 ^{o}C.

After cooling, the samples have been washed three times with distilled water and then with ethyl alcohol –a solution with 70% concentration. The samples have been immerged in a blood plasma similar solution at a 37^{o}C temperature, for one day. Thus an ion exchange is taking place between the solution and the layer of sodium titanate on the surface of the titanium samples that leads to building upon a titanium hydrogel capable of inducing hydroxyapatite-crystallizing nuclei. After 2 days, the solution has been changed with a 1,5 SBF solution (5,6,7). Further, the metal implants have been kept in a thermostat enclosure at 37^{0}C ± 0.3 ^{0}C, during 21days. Periodically, at 2–3 days, the 1.5 SBF solutions have been completed to ensure the losses by vaporization, and especially for completing the losses of Ca^{2+} and PO_4^{3-} ions.

The blood plasma similar solution (SBF) is prepared by dissolving in distilled water of the following reagents: NaCl, KCl, $NaHCO_3$, $MgSO_412H_2O$, $CaCl_2$ and KH_2PO_4 in the needed quantities (table 1). The solution is brought to pH=7,3 with a tris(hydroxymethyl) aminomethane and HCl at 37°C. Natrium azide (NaN3) has been added in the solution in order to stop the bacteria increase (table 1)(7,8,9).

Table 1. Chemical composition of the blood plasma similar fluid (SBF) (mM/l)

	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
Plasma	142.0	3.6-5.5	1.0	2.12-2.6	95-107	27.0	0.65-1.45	1.0
SBF	142.0	5.0	1.0	2.5	131.0	5.0	1.0	1.0

Based on these researches, the coating technology of the biological hydroxyapatite on a titanium support has been achieved and the biological properties have been evaluated in vitro. The titanium samples for the investigations of the biological properties in vitro have been packed in double polyethylene bags and gamma sterilized at 25 kGy radiation dose.

The research of biocompatibility has been carried out on an osteoblast cultures from secondary paths in the presence of implants (10). The in vitro experiments were aiming at testing biocompatibility by sheep osteoblasts cultures in the presence of the composite matrix. For the experiment, isolated sheep osteoblasts have been used and kept in the culture according to the Gallagher protocol (11).

The protocol for isolation and the primary osteoblasts culture consists in fragmenting the collected bones in small fragments which is washed with PBS (Phosphidroxiapatite Buffered Saline) and are put in Petri vessels. The increasing medium DMEM (Dulbecco's Modified Eagle Medium) is added which contains 10% fetal bovine serum. Within 7-10 days the cells from explants occur. After 4-6 weeks the cultures become confluent.

The passage and the secondary cultures are obtained by removing the confluent cells medium and their trypsinization with a trypsin EDTA solution for the detachment of the cells from the support. The detached cells are collected in a 50 ml polypropylene tube that contains 5 ml DMEM (Dulbecco's Modified Eagle Medium) with 10% fetal bovine serum for inhibition of the trypsin activity. It is centrifuged during 10 minutes and the cell sediment is suspended in increasing medium (11,12).

For the experiment osteoblast cells existing in the fifth passage have been used. The testing method used was the one by direct contact (8). The cell suspension has been dropped from the dropper over the tested material put previously in Petri plates. In parallel, witness plates have been prepared where cells have been used only. All the samples have been incubated at 37^oC +/- 2 ^oC in air medium with 5% CO_2 as buffer system for the culture medium. Under these conditions the samples have been kept for 72, 96 and 120 hours, respectively. The evolution of the culture has been monitored on daily basis by electronic microscopy in phase contrast. The experiment has been stopped when the culture confluence was reached.

3. Results

The biological hydroxyapatite coating process by precipitation from blood plasma similar solution is a mimetic process and it is assumed that after implant this continues.

Pursuing the process of obtaining and using titanium implants coated with a superficial hydroxyapatite layer presumes a superficial accurate physical-chemical and then biological determinations of the surface, before implanting in living organisms. The implant is analyzed from the physical-chemical characteristics of the surface viewpoint before implanting in living organisms. The chemical and mineralogical composition of the coated layer is established.

The electronic microscopy scanning images allow a profound analysis of the crystals on the titanium surface. The investigations by electronic microscopy scanning emphasized the presence of a hydroxyapatite crystal layer on the surface of the three titanium samples.

In vitro experiments aimed at testing the biocompatibility by sheep osteoblasts culture in the presence of the composite matrix.

Within the study, in vitro experiments by increasing osteoblasts on implants, the evolution of the culture has been pursued on daily basis, in phase contrasts electronic microscopy. The experiment has been stopped when the confluence of culture was reached. Within this study cells existing in the fifth passage have been used. The cells have been seeded at $2x10^5$ cells/ml initial density and have been left in the culture during 72 hours and then hypericin has been added and the samples were then kept another 12 hours in culture (11). Hypericin colors fluorescent the cells allowing their emphasizing in florescent light. A cell culture under the same conditions, but in absence of titanium implants has been used for checking.

The performed studies had as goal to emphasize the cell morphology of osteblasts in the culture on the biomaterial surface, at the biomaterial- culture medium interface but also on the culture medium surface, at a distance from titanium. A cell culture under the same conditions but in absence of titanium implants has been used for checking. Image of the osteoblasts witness culture has been observed in natural (fig.1) and in fluorescent light (fig.2) light electronic microscopy after 72+12 hours (the sample is kept another 12 hours in hypericin).

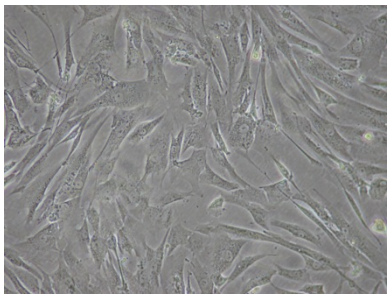

Fig.1. Osteoblasts witness culture in natural light (SEM image).

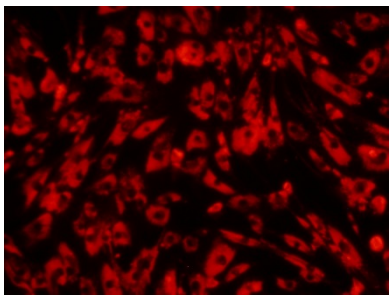

Fig.2. Osteoblasts witness culture in fluorescent light (SEM image).

For comparison the electronic microscopy images in natural light of the osteoblast culture after 72+12 hours on T1 support coated with biological hydroxyapatite after 21 days of immersion in blood plasma similar solution (fig.3), as well as electronic microscopy image in fluorescent light after 72+12 hours both on T1 sample surface (fig.4), and on the culture medium–implant interface (fig.5) for sample T1, after 21 days of immersion in blood plasma similar solution have been monitored.

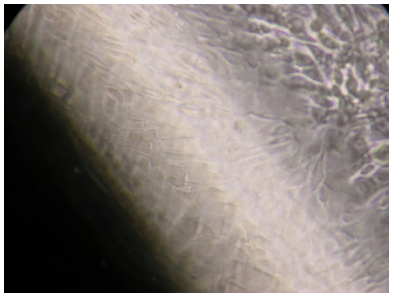

Fig.3. Osteoblast culture in natural light on T1 support after 21 days of immersion in SBF (SEM image).

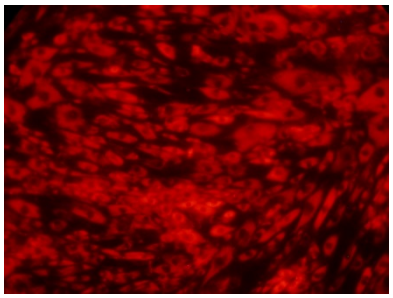

Fig.4. Osteoblast culture in fluorescent light on the on the titanium sample T1 surface after 21 days of immersion in SBF (SEM image).

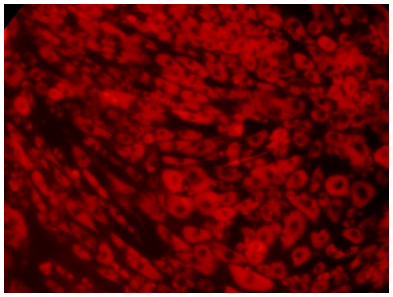

Fig.5. Osteoblast culture in fluorescent light at culture medium–implant T1 interface after 21 days of immersion in SBF (SEM image).

A higher density of cells on T1 support and on the implant –culture medium interface is remarked than on the witness sample. On the implant – culture medium interface a continuous monolayer with osteoblasts is noticed.

In case of cell culture on T2 support the examination has also been made after 21 days of immersion in blood plasma similar solution. In SEM images in natural light (fig.6) of the osteoblast culture after 72+12 hours, as well as in the SEM images in fluorescent light, the cells density on the surface of the implant coated with biological hydroxyapatite (fig 7) as well as its interface with the culture medium (fig 8) is smaller that in case of the T1 sample. Moreover, in case of these implants, the cells showed not only density differences but also morphology changes (cells with smaller size and irregular distribution).

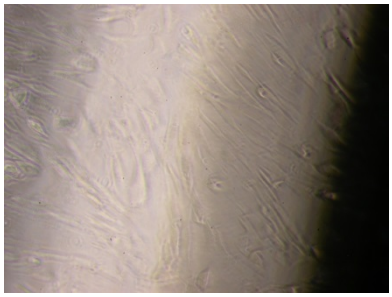

Fig.6. Osteblasts culture in natural light on T2 support after 21 days of immersion in SBF (SEM image).

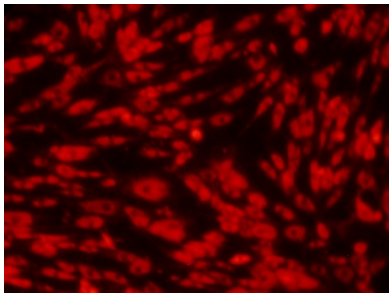

Fig.7. Smaller osteoblasts on the surface of T2 titanium sample after 21 days of immersion in SBF (SEM image in fluorescent light).

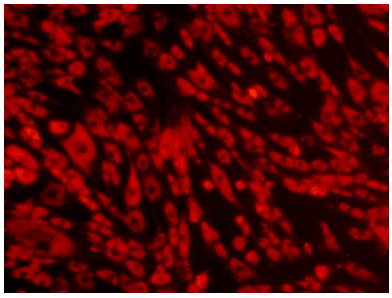

Fig.8. Morphological changes of osteoblasts at the culture medium–implant interface T2 after days of immersion in SBF (SEM image in fluorescent light).

In case of T3 samples, a smaller cell density than in case of the T1 and T2 sample is noticed both on the sample surface (fig.10) as on the titanium –culture medium interface (fig.11). This density is approximately equal with the one of the witness sample. The examination has been made after 21 days of immersion in blood plasma similar solution. The same cell density is also noticed on the electronic microscopy image in natural light (fig.9).

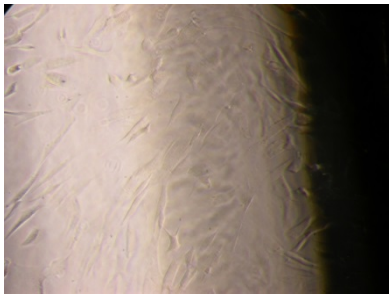

Fig.9. The osteoblasts in natural light on T3 support after 21 days of immersion in SBF (SEM image).

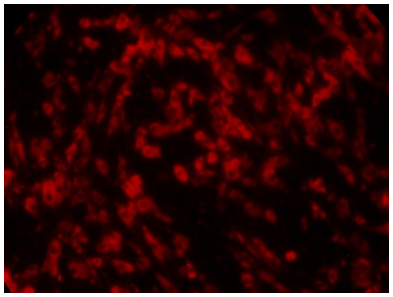

Fig.10. Reduced density osteoblasts on the surface of T3 titanium sample after 21 days of immersion in SBF (SEM image in fluorescent light).

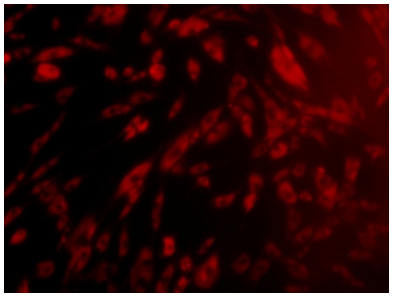

Fig.11. Reduced density and cells morphological changes at the culture medium–implant interface T3 after 21 days of immersion in SBF (SEM image in fluorescent light).

Due to the fact that the biological hydroxyapatite layer coating has been made simultaneously on all the 3 types of implant that is under the same conditions the different behavior is ascribed to the difference in titanium purity

Another study has pursued the osteoblasts culture in the presence of a implants resulted from mechanical processing, and that were not submitted to coating with biological hydroxyapatite layer.

The Sem image of osteoblasts culture in the presence of titanium T1 sample which is not chemically coated with biological hydroxyapatite (fig.12) is studied in comparison with the microscopic image of a T1 sample coated with biological hydroxyapatite (fig.13). As it may be noticed from the electronic microscopy image of the sample, which was not chemically coated with biological hydroxyapatite, the osteoblasts are submitted to a powerful cytotoxicity due to the dissolution of the titanium ions and they die.

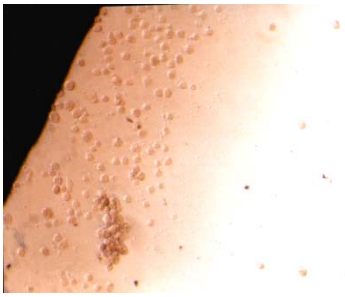

Fig.12 Image in natural light– the presence of osteoblasts on the surface of T1 sample, which is not chemically coated with biological hydroxyapatite (SEM image).

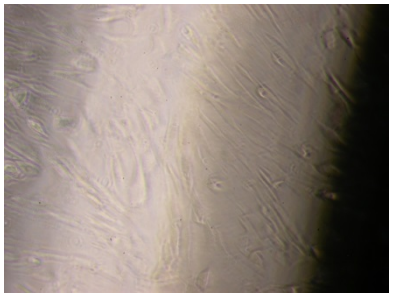

Fig.13 Image in natural light of osteoblasts culture in the presence of T1 titanium sample chemically with biological hydroxyapatite (SEM image).

4. Discussions

Biocompatibility reflects the capacity of a material, inserted in living organisms, including in human body, to be tolerated by this without giving birth to specific defense reactions and at the end of rejection. The biofunctionality of a material represents the capacity of the material to take over mechanical tasks of the substituted bone. No material fulfils simultaneously both criteria and therefore in order to repair or substitute bone in the defects resulted from the resection of tumors or in the areas submitted only to compression stresses, biomaterials coated with bioactive layers are used (12,13). In contact with blood plasma in the superficial layer of these materials passing in solution of the calcium and phosphate ions is taking place and at the interface a silica gel layer is formed which represents the support for biological hydoxyapatite coating that mixes with the collagen generated from osteoblasts existing in the proximity of the layer. A new bone structure is built up thus. It has been found out during the experiments that after 3 months as of implanting, this is so strong so that the action of tensile forces either break implant or the old bone, but never the new built up layer (1).

Whereas the ceramic boactive materials have a smaller bending strength than the bone and they are fragile, for the areas with great and complex stresses, such as hip articulations and of the knee as well as the teeth, metal implants (Cr-Co-Mo stainless steel or titanium) are used. In contact with living tissues these are covering in time with the fibrous layer characteristic to the reaction against foreign body. This increases in time in thickness and leads to the detachment of the prosthesis and hence to its replacement after approximately 10-15 years (14).

This explains the preoccupations either for the achievement of materials to fulfill both requirements (biological and functional), or coating with biologically tolerated material (metals, alloys and plastics) or biologically inert (ceramics based on Al_2O_3 and ZrO2) with thin layers of hydroxyapatite (6,14); thus the support (the sub layer) confers to the prosthesis the needed mechanical properties, and the coating layer confers the biological property and the capacity of chemically react with living tissues, respectively.

In vitro, in the presence of blood plasma similar solution, at 37 ^{o}C an ions exchange takes place between the solution and the sodium titanate leading to building up titanium hydrogel capable to induce structuring of hydroxyapatite crystals nuclei (7,8,13,). This is emphasized by the decrease of Na^+ ions and the replacement with hydronium ions by ion exchange with the surrounding fluid. The concentration of calcium and phosphate ions from the blood plasma similar solution is reduced as consequence of their coating on the titanium gel forming hydroxyapatite crystallization germs (14). Once formed, they grow spontaneously by consuming calcium and phosphate ions from the surrounding liquid, giving birth to hyidroxyapatite crystals similar with those of the bone structure. The consumption of calcium and phosphate ions is also accompanied by a progressive decrease of the pH of the surrounding fluid. In vitro, in order to accelerate hydroxyapatite building up process a solution with higher concentration in the component has been used (1,5 SBF). Hydroxyapatite coating process is proportional with the duration of keeping in the blood plasma solution (6,8,9).

The method described in this study allows coating of a biological hydroxyapatite layer at low temperature on titanium or its alloys support as well as on ceramic implants based on

zirconium dioxide (ZrO_2), by immersion in a liquid similar from chemical viewpoint with the mineral part of the blood plasma (SBF).

The method has at its basis the finding (6,8) of the catalysts role of silanol groups (Si-OH) in coating on material of a biological hydroxyapatite layer (which contains also CO_3^{2-} groups, the structure being thus less stable). If on the surface of the titanium and of its alloys implant Ti-OH groups similar to silanol groups might be achieved, hence coating biological hydorxyapatite from SBF on titanium or its alloys in vitro would be possible .

After implanting bioactive glass in the bone, some authors showed that the bone-implant interface the reactions of new bone structure building up takes place in 12 stages as showed in Table 2 (7,9).

Table 2. Stages of forming reactions of the new bone structure at bone–implant interface.

Crt. No.	**Stage**
1	Implanting the material
2	Building up SiOH bonds on implant surface
3	SiOH+SiOH⟶ Si – O – Si polycondensing
4	Adsorption on the gel surface of Ca +P + CO_3 ions
5	Crystallization of biological hydroxyapatite (HCA)
6	Adsorption of the biological components in HCA layer
7	Macrophage cells action
8	Attachment to stem cells
9	Differentiation of the stem cells in osteoblasts
10	Matrix generation
11	Matrix crystallization
12	Proliferation and increase of bone

Based on this study it is easy to establish by analogy the kinetics of the processes that take place when coating titanium implants with a biological titanium hydroxyapatite by coating from blood plasma similar solution and their implanting in the living organism (table 3).

Table 3. Stages of forming reactions of the new bone structure on titanium implants covered with carbonated hydroxyapatite.

Crt. No.	**Stage**
1	Surface mechanical processing
2	Tina bonds building up on the implant surface
3	Natrium titanate densifying
4	TiOH groups building up ion exchange TiONa ⟶ TiOH
5	Adsorption on the Titanium gel surface of Ca +P + CO_3 ions and HCA nuclei structuring
6	Crystallization of biological hydroxyapatite (HCA)
7	Adsorption of biological components in HCA layer
8	Macrophages cells action
9	Attachment to stem cells
10	Differentiation of the stem cells in osteoblasts
11	Matrix generation
12	Matrix crystallization
13	Attachment to stem cells

The first stages of the process consist of $H3O^+$, Na^+, H^+ ions forming on the implant surface, silanols polycondensing, silica gels formations and forming reactions of biological hydroxyapatite and of the carbonated hydroxyapatite.

The other stages consist of osteoblasts colonization cell reactions, then proliferation and differentiation of cells, building up a new tissue chemically and mechanically bonded to the surface of the implant.

Unlike the bioactive ceramic implants, in case of titanium implants coated with carbonated hydroxyapatite, the processes from stages one to six are taking place outside the body. After implanting the processes continue from the sixth stage and the biological processes are starting (6).

It would be presumable that the duration of processes at the interface to be shorter in this case because building up carbonated hydroxyapatite takes place in vitro, but the difference is small because the duration in situ of the processes at the surface of implant (stages one to five from the table 4) in case of bioceramics is small (under 10 hours).

According to certain authors (15,16), besides the chemical composition of the surface of the implant, the biological response of the neighboring tissue is strongly influenced by the morphology of the surface, this playing an important role in the osseointegration process. The size of the superficial irregularities of the implant affects in different ways the osteoblasts interactions with the surface:

- Lateral size higher than 100 μ allows bone increase in these irregularities. They are important for mechanical fixing (interlocking) of the bone, leading to the increase of the implant stability;
- Lateral size ranging between 1 and 100 μ influences the occurrence, adhesion and orientation to osteoblast cells;
- Lower than 1 μ size influences the chemical stability of the superficial layer (direct contacts and cytoskeletal arrangement), affecting the cell adhesion and morphology; it also influences the biological mollecules adsorption (e.g. proteins).
- Vertical size between 0.05 and a few microns affects cells direction, as well as the communication between them.

The methods studied show the advantage of creating a covalent bond (hence very strong) between the implant and the coated layer. In case of the other methods, the bond between implant and the coated layer is of mechanical nature, therefore weaker (7).

In vitro studies pursued to emphasize cytomorphology of osteoblasts in culture on the surface of the biomaterial, at the material-culture medium interface as well as on the surface of the culture medium, at distance of titanium. A higher density of cells on T1 support and on the T1 - culture medium interface is noticed than in case of the witness sample, a continuous monolayer of osteoblasts being structured on the interface which indicates the bioactivity of the obtained material (16,17,18). In case of samples T2 and T3 the density of osteoblasts is more reduced than ion case of T1 sample and this due to the difference of titanium purity between the three samples.

Analysis of cells culture in contact with the implants coated with hydroxyapatite by immersion in blood plasma similar solution has established the absence of the cytotoxicity and the favorable behavior in the cell medium of thus implant type. The degree of purity of the titanium used as support for the biological hydroxyapaptite coating influences the biological processes at the implant – living tissue interface (fig.3-11)(19,20). Chemical coating of the titanium surfaces with biological hydroxyapatite allows increasing and developing osteoblasts at implant – culture medium interface. Using titanium implants without being submitted to a passivation process by coating with a layer of TIO_2 (by acids attack) or of biological hydroxyapatite by various methods is not recommended (21,22,23).

Titanium implants coating with a nanometric layer of biological hydroxyapatite allows obtaining biologically active implants, capable to chemically react with living tissues.

5. Conclusions

We have thus demonstrated that the biomaterial obtained by hydroxyapatite coating on titanium support in the titanium obtained from blood plasma similar solution is capable to react chemically with the living tissues due to the biological hydroxyapatite nanometric layer formed on

its surface. The objective of this study was also reached: the very high bioactive potential of the biomaterial obtained that allows the development of osteoblasts at the interface with the receptor bed has been emphasized.

Titanium coated chemically with hydroxyapatite is, generally, accepted by the body does not produce reactions. Due to the excellent biocompatibility and the total absence of adverse reactions in the host tissues as well as the absence of any allergic reactions, these biomaterials are very suitable for different stable medical-surgical applications.

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