The CGF. A therapeutic proposal for regenerative medicine

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Regenerative medicine is one of the biggest targets of today's rehabilitation therapies. The best tissue stimulation is derived from the autologous GFs which induce regeneration. For this purpose, many products and techniques have been used (i.e. Tissucol, PRP, PDGF, PRF, etc.). Though, none of these systems proved to be fully successful for an appropriate biostimulation. This is due to the fact that none of the above mentioned techniques exploit the regenerative potential of the whole blood. The CGF technique envisages the use of all the separated blood phases which can be disposed of individually in order to obtain the biostimulation of the related cells or tissues

The desire and the need to be able and reconstruct portions of lost or damaged tissue has always been one of the most greatly studied therapeutic aspects of modern medicine. In dentistry, with the coming of the GBR, there has been a significant input in the search for materials and growth factors, applicable to bone regeneration techniques (bib 1-29). We therefore went through several techniques, mainly using different types of both natural and synthetic materials to construct both the membranes and the cavity fillers. All sorts of materials have been used: from Goretex to pericardium, hydroxyapatite, organic glass, tricalcium phosphate, polyglycolic acid, animal bone and human bank bone, and many others. They all aim at bio-stimulating or osteo-inducing bone regeneration, but their best application is to be used as fillers. In fact, all these materials, which represent the history of the GBR, have something basic in common: they are not alive. This could seem irrelevant, but in terms of real osteoinduction or bone regeneration, it becomes fundamental. In regenerative medicine there are many factors involved in this process and rely mainly on the biochemical and hormonal metabolism of each patient. The factors involved in helping tissue regeneration are:

STEM CELLS:

They respond to a scale of proliferative power with various potentials according to the state of differentiation, and can be classified as:

- Progenitors of other cells
- Undifferentiated
- Non specialised
- Unlimited or prolonged proliferation

When appropriately stimulated, the stem cells can differentiate and specialise, and therefore can be classified in:

- **Totipotent**
- **Pluripotent**
- **Unipotent**

The unipotent stem cells are present in blood.

NATURAL LOCAL MODULATORS

The most widely known local modulators produced by bone reshaping and stimulation are:

Insulin-like Growth Factors (IGFs)

Insulinlike growth factors are hormonedependent polypeptides and can be divided in IGF-I and IGF-II. They show a high concentration in periosteum, in the fibrous callus of fractures, in the ectopic bone induced by the demineralised bone matrix. They are produced by the bone cells, but can be incorporated in the calcified matrix and released during re-absorption.

They mainly exert their effects on osteoblast precursors, stimulating their differentiation and proliferation, but also on the osteoblasts themselves, which are stimulated to replicate. They also promote the production of type I collagen and bone matrix synthesis, helping to accelerate the healing process.

Fibroblast Growth Factors (FGFs)

These are a large family of polypeptides (from FGF-1 to FGF-18) and the most important are FGF-a (acid) and FGFb (base), also called heparin-bound growth factors.

They contribute to bone healing after fractures, to the development of the vascular, nervous and skeletal systems and in a variety of normal and neoplastic tissues.

They help angiogenesis, chemotaxis and mitogenesis, stimulating the growth of fibroblasts, myoblasts, osteoblasts, endothelial and neuronal cells.

Cytokines

The cytokines, especially type IL1 and TNF-a, are powerful stimulants for bone reabsorption:

- IL-1 acts directly on the bone where, through the activation of the transcription factors NF-kB, it induces the synthesis of other bone reabsorbent substances, such as the IL-6, TNF-<, and PGE2;
- IL-6 and TNF-< not only do they stimulate bone re-absorption, but also further osteoclastic cell replication (osteoclastogenesis);
- the PGE2 on the one hand mediate the bone re-absorption induced by the IL-6, and, on the other hand, promote the recruitment of the cells in the osteoblast line, stimulating collagen synthesis.
- the VEGF (Vascular Endotelial Growth Factor) stimulates the growth of

new blood vessels. It is produced by the peripheral circulatory system cells (macrophages and T cells) but especially by platelets. It is directly involved in the control of the behaviour of the endothelial cells, particularly in their proliferation, migration and specialisation. This simple cytokine is just enough to stimulate angiogenesis.

Bone growth factors (GFs)

It has been highlighted how bone regeneration takes place under the systemic influence of hormones such as Parathormone, Calcitonin and vitamin D etc., which regulate the new bone fixation and reabsorption process.
The most active factors are codified as BMPs (Bone Morphogenetic Proteins). They stimulate and mediate the growth of the target cells, through a surface cell binder-receptor interaction (Andreana e Ciancio 1993).
The growth factors are present in tissues or parts of tissues, i.e.:

- in blood and plasma,
- in the bone matrix, where they play an important role in the new bone morphogenesis, reorganisation and reshaping, as well as bone healing.

Insulin-like growth factors

The insulin-like growth factors (IGF-I and IGF-II) or somatomedin, stimulate the activity of the osteoblasts by which they are produced and increase collagen production.

Osteoprotegerin (OPG)

The Osteoprotegerin (OPG) is a cytokine from the family of the Tumour Necrosis Factors that, unlike the TNF-oc, has a powerful action in inhibiting the osteoclastogenesis;

Transforming Growth Factors (TGF)

The Transforming Growth Factors (TGF) include a super-family of molecules responsible for the control of many aspects of cell functions. They are synthesised by the platelets, macrophages, endothelial cells, keratinocytes and chondrocytes, the TGFs-ß are mainly expressed by mature, fully-active osteoblasts, both during the bone growth and development and during the healing of fractures. Among these factors, the TGF-p plays a fundamental role in the growth and differentiation of many cells, including the osteoblasts. Its production in the osteoblasts is stimulated by the vitamin D, PTH, estrogens and testosterone. Furthermore, this factor inhibits bone reabsorption, preventing the formation of the osteoclastic precursors and stimulating the apoptosis of mature osteoclasts;

Bone Morphogenetic Proteins (BMP)

The Bone Morphogenetic Proteins (BMP) induce the pluripotent cells to differentiate into cells able to produce bone and cartilage.
They are expressed during puberty, but also in the bone callus formation after fractures, and locally after the implant of micropatterned substrates. Furthermore, they are involved in the morphogenesis and development of many other tissues and organs, such as hair follicles, heart, kidneys, eggs, prostate and, most of all, are involved in the morphogenesis of tooth tissues.

Fibroblast Growth Factors (FGF)

The Fibroblast Growth Factors (FGF) play an important role in bone regeneration and development and in the fracture healing process. Their main task is to induce bone angiogenesis, which is a critical moment for the formation of bone tissue.

LOCAL SYNTHESIS MODULATORS

The best known local synthesis modulators, produced for the stimulation and reshaping of bone, have been the subject of a great deal of research. Many different systems for the preparation and concentration of the growth factors have been developed so far, which we will list hereby:

Exhisting technologies:

- **Fibrin glue** (Tissucol Baxter)
- **Platelet concentrate** (cPRP, Marx 1998)
- **PlateletRich Plasma** (PRP)
- **PlateletRich Growth Factors** (PRGF, E. Anitua 1998)
- **Platelet-Rich Fibrin** (PRF, J. Choukroun, 2001)
- **C.G.F.** (Concentrated Growth Factors 2006, IAIO)

Fibrin glue (Tissucol Baxter)
The human fibrin glue is atoxic thermaltreated biologic adhesive which is highly tolerable. The glue contains both fibrinogen and factor XIII (re-established at 37° with a aprotinin solution which helps slowing down reabsorption inhibiting local fibrinolisis). The bovine thrombin is re-established in a calcium chloride solution, in a concentration of 4 U.I./ml or of 500 U.I./ml. The solutions, kept at 37°, are mixed to obtain the right fibrin glue at the time of its application. The two components are mixed with a double syringe called duploject which will make the 2 component react once the needle is exposed. The mostly used fibrin glue at present is the Tissucol by Baxter. The fibrinogen concentrate is obtained through repeated steps of thermochemical precipitation and the concentrations of fibrinogen and factor XIII are very high. The thrombin solutions are prepared with human plasma (bib 30-49).

Platelet-Rich Plasma (PRP)
The platelet concentrate obtained from the patient's blood, allows for the use of autologous growth factors (PDGF, IGF-I, IGF-II, TGF-f5), which are neither immunogenic nor toxic, and which can accelerate the normal bone regeneration processes and increase both the quality and quantity of the newly-formed bone. When the platelet concentrate, set up in the

form of a gel, is mixed with the filling material, (the best filler is the autologous bone) we therefore obtain a graft tissue with optimal characteristics, which is in theory far better than autologous bone alone due to its ease of stabilisation and better mineralisation times. The technique envisages the collection of approximately 60 ml of venous blood from the patient, yielding, within 45 minutes, a platelet concentrate through two separate centrifuge phases. The interim product is a Platelet-Rich Plasma (PRP).

- In order to obtain the PRP it is necessary to use a specific laboratory testing equipment and to be assisted by a haematologist.

Once the final platelet concentrate has been obtained (PRP) it is activated to form the graft gel, by adding 80 mM of calcium chloride and Botropase (bib 50-162). The PRP is, therefore, a concentrate of platelets, whose destruction releases the growth factors called *Platelet Derived Growth Factors* (PDGFs) which induce the osteoneogenesis. They promote angiogenesis and act on the osteoblast precursors, on which they induce a significant mitogenic action. They increase the number of cells in the osteoblast line, are able to induce the osteoblasts themselves to cell replication and collagen synthesis, but their differentiation and morphogenetic function with regards to the bone tissue is undoubtedly less than other growth factors. Actually, according to the international bibliography, the bone growth induced by the PRP is 10% of the applied volume so, in spite of its biologic potential, the PRP has got a rather low osteoneogenic performance (Malchiodi 2001, CED Rome, bib. 163-166). For this reason, other technologies have been developed, such as the PRF.

Platelet-Rich Growth Factors (PRGF, E. Anitua 1998)

The PRGF is the result of the centrifugation of venous blood which is located under the Buffy Coat and is taken with a pipette. The PRGF, mixed with biomaterials, buffy coat or directly used in situ, enables the biostimulation of the tissue that needs to be regenerated, giving more power to the local healing action (bib 167-217).

Platelet-Rich Fibrin (PRF, J. Choukroun, 2001)

It is obtained from fresh blood taken from the patient's vein.

According to the protocols described by Choukroun et all from 2001 (bib 214-257), in order to obtain the PRF, we simply need to centrifuge the blood to separate its components. As PRF is an unchanged blood product, it can be developed in the dentist's cabinet, as long as the centrifuge is certified for this use. The PRF obtained is a fibrin-rich dense gel, resistant to traction and tear. It does not need to be covered and can act as a membrane. The PRF works as a biostimulator on the receiver tissue. A significantly appreciated effect of the PRF is its analgesic, antalgic and antiinflammatory action. The PRF is developed by centrifuging the blood for approximately 12 minutes at 2700 revs./min. and, once separated from the other blood components, it is temporarily stored in a refrigerated environment at a constant temperature between 12 and 15°C.

C.G.F. (Concentrated Growth Factors 2006, IAIO)

As we believe in the extraordinary regenerative power induced by the blood, and we know that all the necessary components for regeneration are free and circulating, we investigated on how to use all the healing and regenerative characteristics, and not only some of its parts as proposed by the previous protocols. Unlike the PRP, PRGF and PRF, the CGF is a therapeutic protocol obtained through the separation of the venous blood, subject to a fixed temperature, with a rotor turning at alternated and controlled speed and always accelerating below RCF300.

The CGF is characterised by 4 phases:

1. a superior phase represented by the serum (blood plasma without fibrinogen and coagulation factors),
2. an interim phase represented by a very large and dense polymerised fibrin block
3. a liquid phase containing the GFs, white line cells and stem cells waiting for stimulation and to differentiate into specialized cell types
4. the lower red portion is a viscous, dense, plateletrich coagulation (Fig. 1, sample)

The phases and their components are as follows:

Fig. 1: blood sample after CGF centrifugation: serum, buffy coat, GFs and stem cells, clot

1. SERUM:
The serum is the lightest and most liquid part of the blood.
It is fundamental for our technique because it represents the liquid able to amalgamate all the grafts and supplies many biochemical components and activators. It is fibrinogen-free and has only a few cells. It should be kept cool and mixed quickly in order to avoid denaturing the proteins. It is clear straw yellow in colour and consists of:

- 92 % H_2O
- 7 % proteins, mineral salts, CO_2:
  - Proteins: albumin, antibodies
  - Nutrients: glucides, amino acids, lipids
  - Enzymes
  - Hormones
  - Inorganic electrolytes

The serum is used to wash the cavity, to cover and protect all the regenerated portions.

2. FIBRIN Buffy Coat:
Thanks to the calibrated centrifugation carried out with the Medifuge phase separator (Silfradent, Italy), through the polymerisation of the fibrinogen molecules (FG) the fibrin block is obtained as comprising three-dimensional polymer networks with interwoven fibres, all collected in a single phase in the form of gel. During the polymerisation, the fibres' diameter grows until the end of the reaction (fig. 2-3).
This concept explains why it is important to set up the equipment specifically, guaranteeing the maximum exploitation of the blood's potential by controlling the following settings:

- Speed
- Temperature
- Time
- Acceleration and controlled speed
- Gravitational acceleration of approximately RCF200

The development and growth of the fibrin gel block during the centrifugation, and especially during the polymerisation phase, allows for a volume growth of the chains in all directions (fig. 4).
In this way, many corpusculated components are dammed, determining numerous therapeutic actions, such as:

- plasma and platelet cytokines: repair, anti-inflammatory and pain-killing effect during repair (TNF-a);
- platelets: transmission of the signals and release of the GFs. The most important are the PDGF-BB, TGFI3-1 and IGF-1 (fig. 5).

We therefore obtain significant volume fibrin gel blocks with excellent resistance that can be used as:

- cavity fillers
- membrane supports
- autologous membranes
- particles to be mixed with ano-

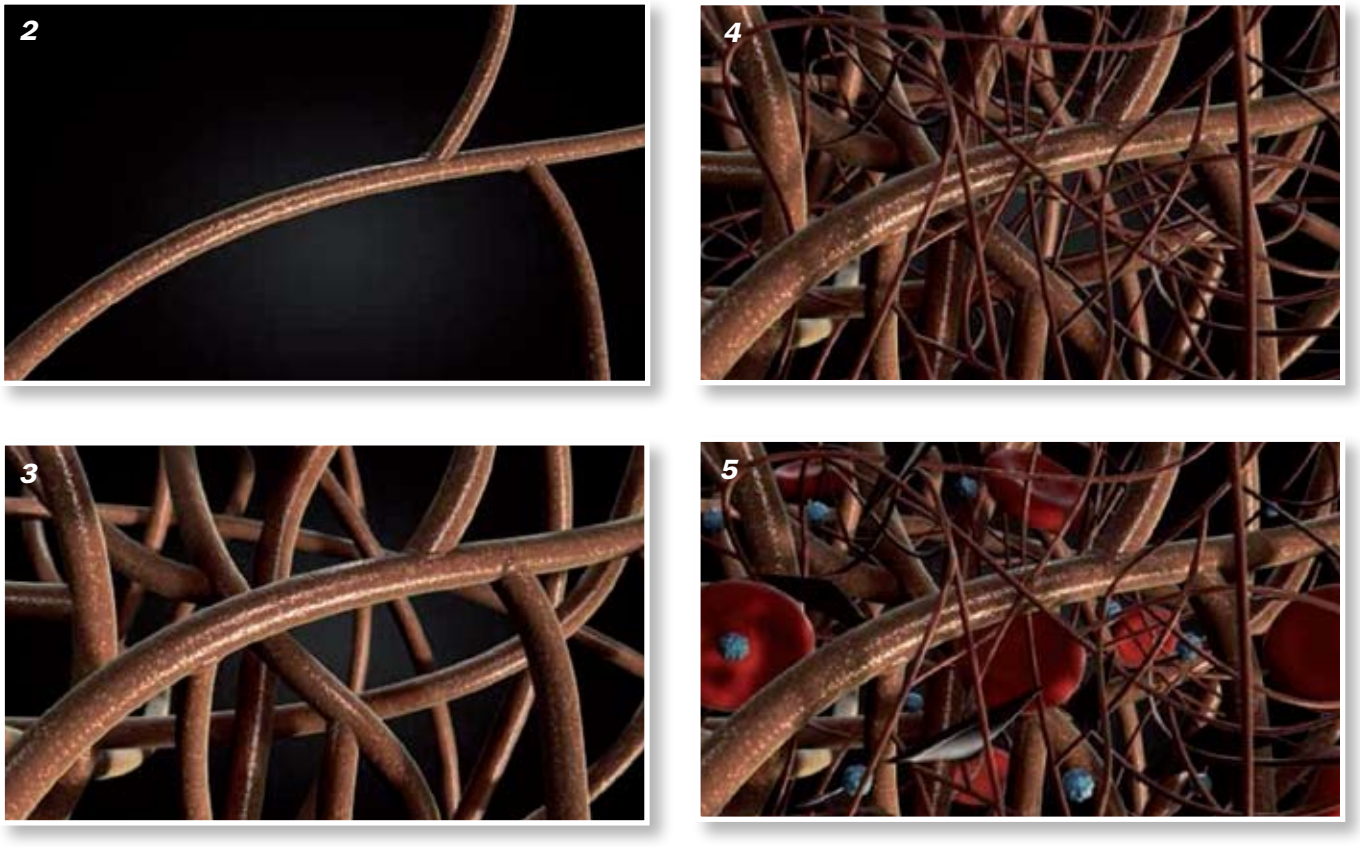


Fig. 2-5: CGF chart: this sequence shows how the buffy coat molecule in the CGF is extremely ordinary and help wedging the anticorpal molecules, platelets, white, red and stem cells

ther filling material.
This translates into simplified work and a high power for regenerative induction and a greater versatility of use of the fibrin block, ranging from the use of the whole block to the particles or membrane.

3. The Growth Factors and the unipotent Stem Cells
located just below the buffy coat and above the dense clot portion. This phase can be aspirated with a pipette and mixed with autologous bone in order to obtain an extremely performing activated graft.

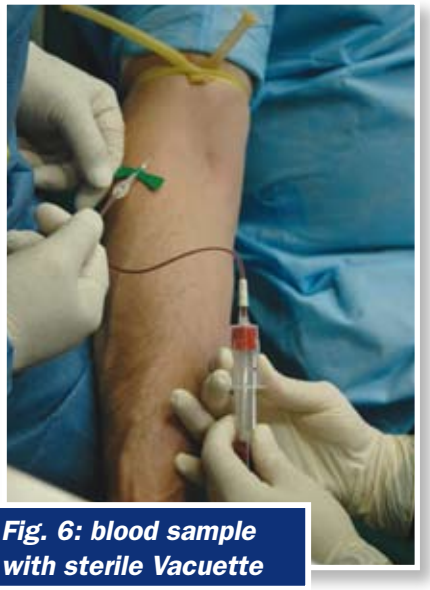


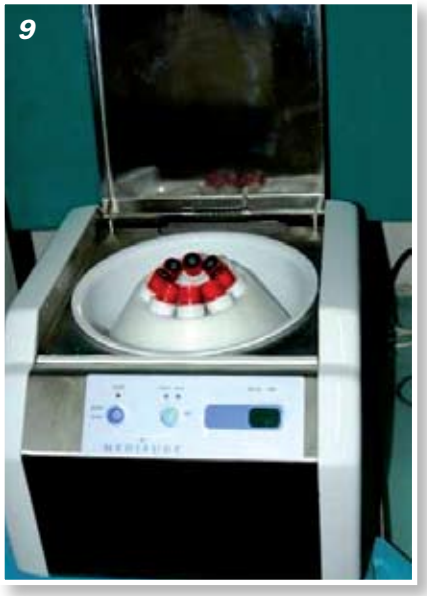
Fig. 6: blood sample with sterile Vacuette

4. COAGULATION
In the CGF technique, the red phase consists of concentrated red and white blood cells, platelets and clotting factors. It looks like a dark reddish dense gel and can be used pure or mixed with fibrin particles and/or autologous or heterologous bone when filling very large cavities. We can therefore assess that the CGF in regenerative medicine should therefore be conceived as a multifactor stimulation system. In fact, all the phases and components are used according to specific requirements. This versatility and multipurpose application make it stand out from all the other techniques proposed so far.

MATERIALS AND METHODS

In order to obtain the CGF, we begin by taking a venous blood sample using a 21 x ¾ gauge butterfly vacuette needle and a vacuumpacked Vacuette 9 ml Z Serum Clot Activator (Greiner bio.one, Austria, fig. 6).
Once filled, the test tubes are quickly placed into the rotor of the ***Medifuge (Silfradent, Italy) centrifuge accelerator***, without shaking them (fig. 7).
This has exclusive characteristics with regards to:
- mechanical structures and characteristics, such as, for example, the monolithic sterilisable rotor (fig. 8)
- calibrated angled test tube (fig. 9)
- working temperature
- disinfection of the rotation chamber
- dynamic characteristics
- settings: start, acceleration, speed and brake for the fluid to be centrifuged
- automatic, closed lid disinfection.

All this permits to obtain more greatly differentiated components right from the test tube.
- After approximately a 13 minute rotation, the serum is separated from the other phases of the



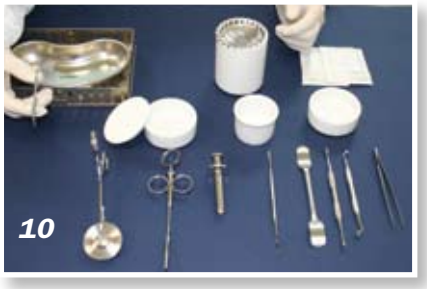


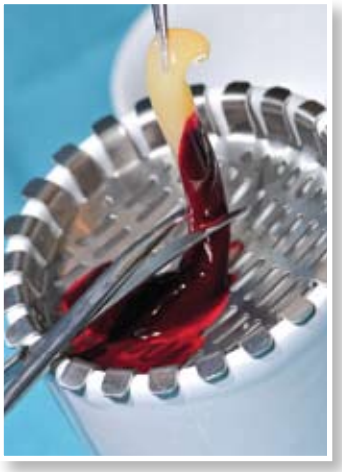
Fig. 7: Medifuge phase separator (Silfradent, Italy). This device will guarantee the control of the right speed and acceleration to separate the blood without damaging its components
Fig. 8: Medifuge's monolithic rotor. It is dismountable for sterilisation.
Fig. 9: Medifuge with blood samples in place
Fig. 10: set of sterile dappens to separate and store the blood phases obtained with the Medifuge

CGF and stored in a specific sterile dappen (fig. 10).

- The fibrin phase is separated and stored in diluted antibiotic solution (Lincocin 600 mg).
- The initial portion of the coagulation containing the GFs and the stem cells are immediately stored in the dappen provided
- The coagulation, which is rich in red blood cells and platelets, as well as iron, calcium and other fundamental components, is prepared to be used for the preparation of fillers, for mixtures of biomaterials or autologous bone taken for osteotomy (fig. 11).

The fibrin block, separated from the red phase, is prepared to be transformed according to needs: direct cavity graft, shaped membrane with the use of the specific forceps provided (fig. 12-13), graft particle to be mixed with biomaterial or living autologous bone (fig. 14).

- A specific process is necessary to obtain an autologous CGF graft for large cavities. In this case, the fibrin block is cut into particles of approximately 1-2 mm while the clot is fragmented and mixed with the fibrin particles, with fresh blood and further graft material, best if autologous bone. To increase the softness of the mixture, some serum can be added.



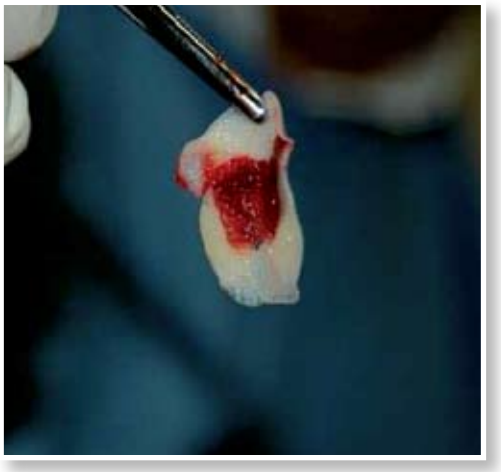


Fig. 11: Clot separated from the fibrin block. The former is stored in a sterile dappen which is closed to avoid the excessive oxidisation of the red cells and the emoglobine and to avoid to over-breaking the platelets. Fig. 12: the membrane obtained by using the forceps provided

This is all mixed and homogenised mechanically in the specific ***Round Up device (Silfradent, Italy)*** for approximately 6 seconds (fig. Round Up 15).

This dense and particularly adhesive paste is inserted into the cavities or bone defects, proving to be extremely mouldable. Then is all covered by applying the CGF membrane obtained by squeezing the fibrin blocks with the forceps provided. CGF membranes are used to cover wounds or reconstructed areas, which can stick together thanks to their adhesive power and, thanks to their elasticity, can be sutured. At the end of the surgery, you can brush the wound with some serum.

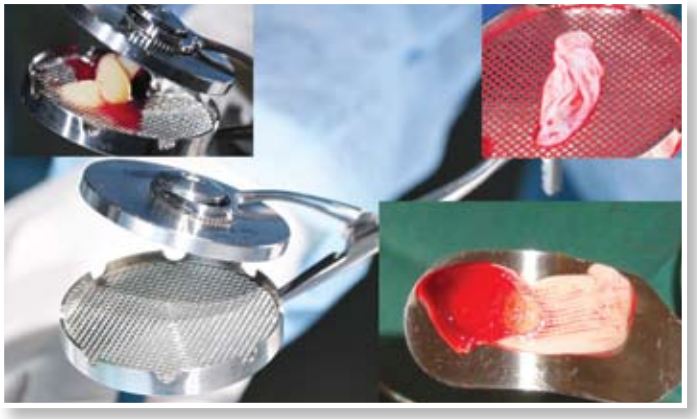


Fig. 13: membrane after forcep shaping and placed over the proper spatula for an appropriate an easy positioning. Fig. 14: fibrin particles mixed with grafting material. The mix of these 2 component with the Round Up takes only 6 seconds. Fig. 15: Round up