VISSAGRAN

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FUNCTIONAL APPROACH

The maintenance of joint activity is directly linked to its use: a fracture with prolonged impediment of joint mobility demands a significant recovery period that grows longer with age. This is due to the fact that movement generates a constant turnover of the synovial fluid (SF) and the extracellular matrix of the articular cartilage (ECM).

1. DESCRIPTION OF BOTH STRUCTURES

Synovial Fluid:

Its constant production stems from the synoviocytes on the articular capsule. SF plays several major roles in ensuring joint preservation:

a) Cleansing and nutriment of the ECM. The articular cartilage is an avascular tissue in constant renewal that requires nourishment to be able to clean the detritus that it produces. This function is carried out in the most active part by the synovial fluid and, on the bone spectrum, by the subchondral bone.

b) Edge lubricant that prevents the impingement of the joint capsule caused by flexing of the articulation

c) Maintenance of joint space, which prevents the impact between the articular facets under great pressure.

The SF is rich in hyaluronic acid (HA), whose viscoelasticity properties, based mainly on the concentration, polymerization and magnitude of its molecular weight, enable the liquid to put into practice the aforementioned effects (Gibbs D et al., 1968; Hamerman D et al., 1966).

d) Limit lubricant exerted in conjunction with a thin amorphous layer of a superficial protein complex of the articular cartilage.

This association is the HA-lubricin integration. It would have a basic function of the boundary lubricants: granting low friction and wear protection to the shearing surfaces (Hui AY et al., 2011).

As it has been proven experimentally on rats, on the shearing wear-down both components need to be present, since the HA alone does not carry out said function (Teeple E et al., 2011).

The physical bond between HA and lubricin is relatively weak, and it can be separated due to normal friction and shearing efforts. The compression provokes diffusion of the nominally free HA to physically trapped in the interface of the increasingly constricted collagen pore network. The mechanically trapped HA-LUB complex now acts as an effective (chemically bound) boundary lubricant —reducing the friction force slightly but, more importantly, eliminating wear damage to the shearing surfaces of the cartilage (Greene GW et al., 2011).

Human and animal studies have revealed that HA's molecular weight bestows it of viscoelasticity properties that depend on the concentration and the polymerization grade within the synovial fluid (Gibbs DA et al., 1968; Hamerman D et al., 1966).

The HA on the synovial fluid varies between 2 and 6 million δ.

The concentration of HA synovial fluid varies among species and between the articulations of each individual (Balazs EA, 1967; Balazs EA, 1986; Auer JA, 1980).

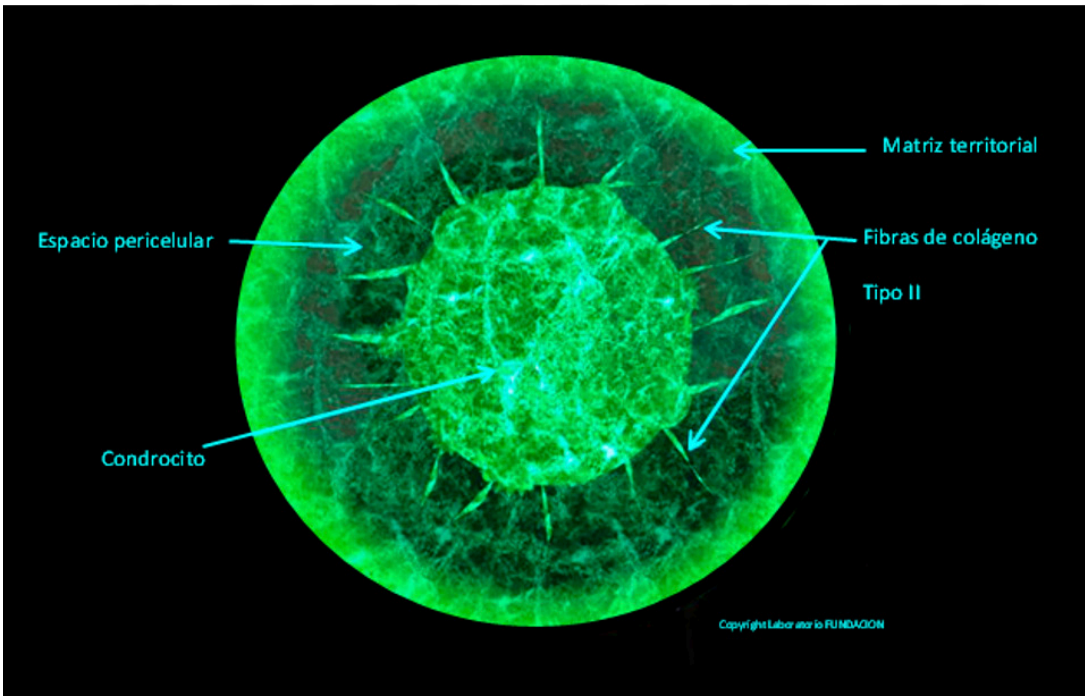
The normal equine synovial fluid reference values vary between the range of 0.33 to 1.5 mg/ml, depending on the researcher and the applied technique.

Articular cartilage:

Chondrocyte

The cell unit of the cartilage. Even though it represents a low percentage of the tissue, it is metabolically very active individually. It is located in a pericellular space adhered to the walls (territorial matrix) through collagen fibers that convey physical transformations (mechanical and electrical), besides chemical influences from the medium. This information enables it to maintain a balanced ECM in constant remodeling (synthesis and degradation). It is nourished by the synovial fluid and the subchondral bone. It works almost in a state of anaerobiosis.

See representation in the next page.

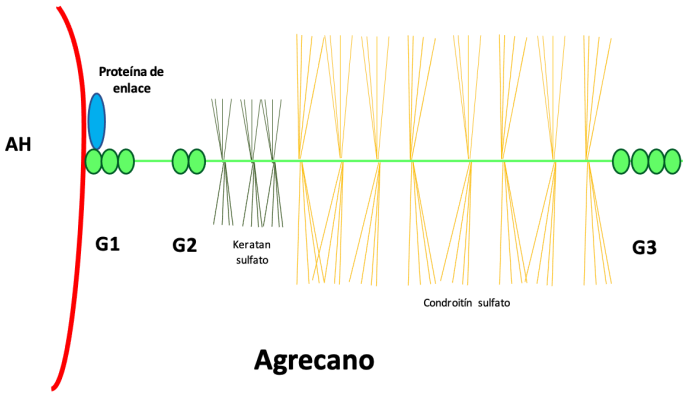


Extracellular Matrix (ECM)

It frames the space between the chondrocytes, and its composition can vary in several aspects: closeness to the cells, depth, articulation, age, among others. The two most abundant elements of its composition, of which its function depends on, are water and the macromolecular groups containing it. Two of these components are key:

1) The collagen fibers that form the matrix skeleton binding the chondrocyte to it, as previously seen
2) The proteoglycans, which are made up of the aggrecan molecule, the binding protein and an HA chain. Ensembled outside of the chondrocyte, these complexes intertwine with the collagen fibers (Wight T, 1987). They provide intense negative charges to the matrix that carry SF cations (mostly Na) that attract water. This hydrated and viscous gel provides the osmotic properties required for the compressive resistance resilience[1] that characterizes the tissue (Kisiday J et al., 2002).

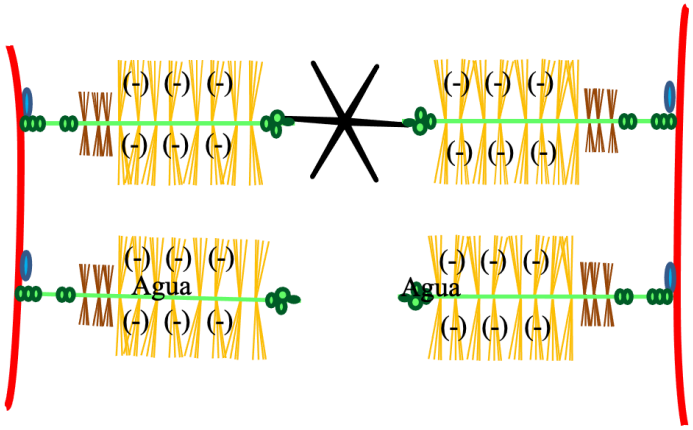
Figure 01



In Figure 01, the HA is shown in red (a single red line that forms the base). The components of aggrecan are: the central protein with its three domains (G1, G2 and G3) marked in green, the glycosaminoglycan chains marked in brown along the central protein (between G2 and G3). The binding protein marked in blue. Aggrecan binds to HA through its ternary complex N-terminal G1 domain with the binding protein (Inspired by Aspberg A., 2012).

1. Resilience: the ability of a substance or object to spring back into shape when the force causing the deformation ceases.

Figure 02

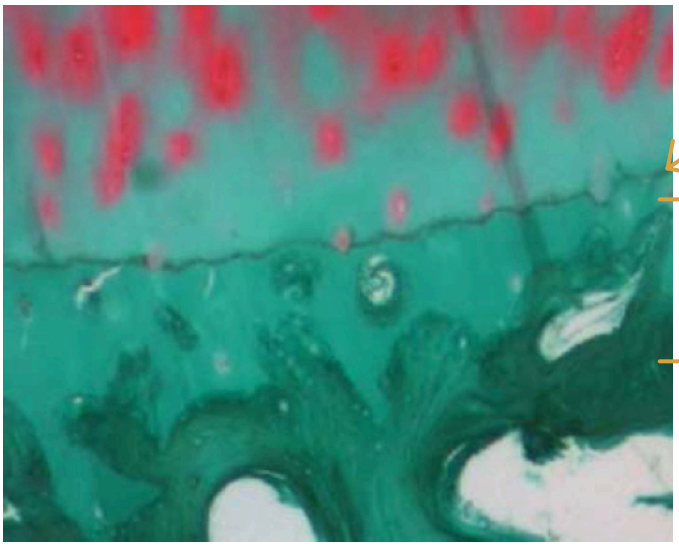


In Figure 02: G3 domain-mediated organization of the extracellular matrix aggregate. The model describes how interaction with a protein (tenascin in black), through the G3 domains, can cross-link proteoglycan aggregates and organize the extracellular matrix. The charges (-) are the executors of the retention of cations and water (Inspired by Aspberg A., 2012).

The molecular weight (size) of the HA in the cartilage diminishes with age, but the quantity increases. This can also be perceived in the aggrecan complexes of the ECM, which increase from 1% in newborns to 10% in adult animals. This is possibly due to the accumulation of aggrecan residue (G1 domain) and link proteins (Holmes MW et al., 1988).

Chondro-osseous junction

In a brief description, this transition zone is made up of:



TIDEMARK: basophilic line marking the front mineralization of the MEC.

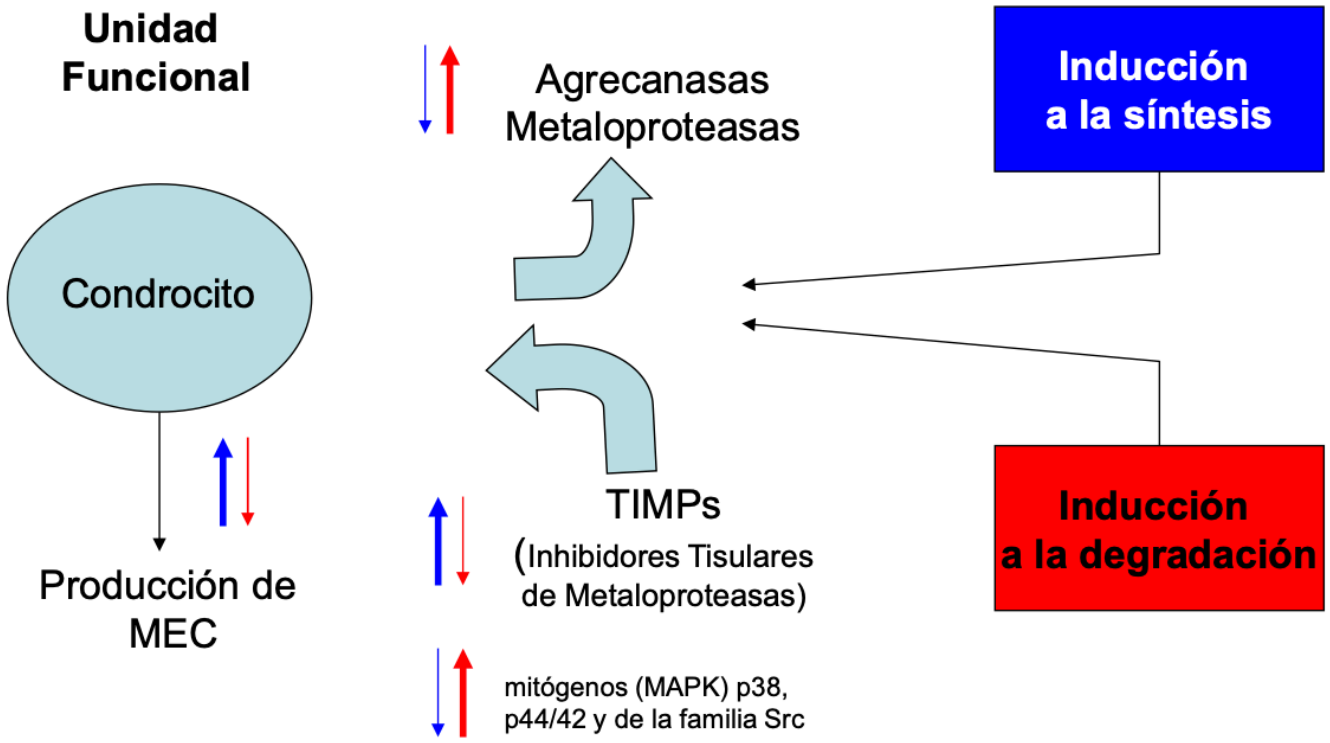
CALCIFIED LAYER: they are chondrocytes in a mineralized ECM. Its function is to anchor the synovial cartilage to the subchondral bone. It is 10 times less rigid than bone.

SUBCONDRAL BONE: its function is to absorb mechanical loads applied to the joint and supply the cartilage with nutrients and oxygen.

(Taking from: Pesesse L et al. ; Osteochondral plate angiogenesis; Joint Bone Spine; 78 144–149; (2011))

2. PHYSIOLOGY OF THE CONSTANT RENEWAL OF THE TISSUE:

The balance between two forces, synthesis (anabolism) and degradation (catabolism) is established by the sum of physical and chemical information that the chondrocyte receives.



In this regard, factors such as IGF-1 (similar to insulin 1) and the TGF- β (transforming growth factor), as many others, stimulate the ECM production. On the other hand, the VEGF (vascular endothelial growth factor), the interleukins 1 (β; α), the TNF- α and others, provoke the opposite effects (Carlevaro MF et al., 2000; Troeberg L and Nagase H, 2012.)

3. AN APPROACH TO THE COMPREHENSION OF OSTHEOARTHRITIS/OSTHEOARTHROSIS (OA)

On a workshop sponsored by the American Academy of Orthopaedic Surgeons, the National Institute of Arthritis and other institutions, the OA (osteoarthritis) was redefined as:

"Group of overlapping distinct diseases that may have different etiologies but similar biologic, morphologic, and clinical outcomes".

The development of the disease not only affects the articular cartilage, but also involves the whole articulation.
Brandt KD et al. (2008) also consider arthrosis as the result of a disparity between the load or tension applied to the articular cartilage and the cartilage's capacity to carry said load. This can be due to:

- **An increase of the load.**
- **A weakening of the cartilage.**
- **Defective subchondral bone.**

It is worth mentioning the applicability of this approach to sport animals. The same authors mention that the main physical distress during the arthrosis process is pain and the motion-restrictions imposed because of it, considering the neo-innervation accompanying the neo-vascularization of the subchondral bone an apparent origin.

From a biochemical-cellular point of view, the processes would lead to a functional discrepancy between the synthesis and the degradation. In the process chondrocyte clones are generated which, as the received information —via physicochemical pathway— sustains this discrepancy (originated in physical processes, such as deficient stance, non-recuperated traumas with new articular demands, excessive articular stress or specific diseases), keep transforming.

Therefore, we have:

a. Synthesis stage: where, early on, the chondrocyte is trying (partly accompanied by the synoviocyte) to produce more that it needs. The example is the increase of synovial fluid, but with greater fluency due the decrease of HA in quantity and quality.
b. Fibroblastic stage: where collagen production increases (partly defective).
c. Degenerative stage: an excess of metalloproteinases is produced. First those who attack mainly the aggrecan are expressed, and then collagenases increase, which remarkably aggravate the situation.

A chondrocyte group located through the tidemark (see chondro-osseus junction) causes its duplication and accelerates the calcification of the ECM. The neo-vascularization and neo-innervation of the subchondral bone and the synovial membrane are early complications considered the root of the pain (Brandt KD et al., 2008.) Summing up, the functional change of the chondrocytes of the synthesis program and the anti-genesis phenotype towards the catabolic program and the pro-angiogenesis phenotype are characteristic of the osteoarthrosis pathology and the aging of the chondrocytes (Gerber HP et al., 1999; Harper J and Kalgsbrun M., 1999; Thomas M H., 1999; Zhang E et al., 2013.) Articular stress factors and age also reverberate on the changes of the synovial membrane (Sagiroglu A O, 2012.)

THERAPEUTIC IDEA

On a questionnaire given to healthcare professionals specialized in knee OA, three premises that any intra-articular drug for osteoarthrosis should have, based on the harvested evidence, were established:

- **Lubricant function increase.**
- **Inflammation interruption.**
- **Damage recovery.**

The analgesia should be obtained in a lasting way by achieving these three goals.

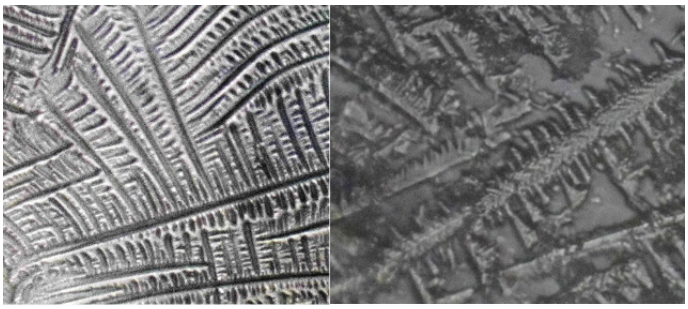
DESCRIPTION

VISSAGRAN is an aqueous solution of sodium hyaluronate highly purified. Its molecular weight is 2.4 m δ. In it, micronized stanozolol (active substance of ESTROMBOL®) is integrated. Each milliliter contains: micronized stanozolol 5 mg; sodium hyaluronate 5 mg; inactive substances and water for injection q.s. 1 ml.

PHARMACOLOGICAL CONCEPT

To integrate the active substance, stanozolol, to a natural matrix of the synovial components; to achieve that, hyaluronic acid is chosen. The first one checks the three aforementioned premises, while the hyaluronic acid is prepared to fulfill the following goals:

a) Being an internalized vehicle for the amorphous powder that, even though micronized, would mitigate any erosion until absorption of the active substance on the articulation.



Hyaluronate image before (a) and after (b) internalization of the active principle (View 40x)

b) Immediately improving the rheological functions of the synovial fluid, until normal synoviocyte activity is restored.

c) Working, to some extent, as an anti-inflammatory agent.

Regarding the fulfillment of the three premises by the active substances, the literature mentions the following: Sodium hyaluronate has been classified as a SYSADOA (symptomatic slow acting drugs for treatment of osteoarthritis) by international specialized organisms such as OARSI (Osteoarthritis Research Society International), ACR (American College of Rheumatology) and ILAR (International League of Associations for Rheumatology).

The viscoelastic solutions of HA have rheological properties recuperation (elasticity and viscosity) of the altered synovial fluid as a goal, re-establishing the homeostasis in the articulation, relieving the pain and amplifying joint motion.

The HA has biochemical activities that are different from its physical properties. It is a powerful inhibitor of the leukocytic activity, and prevents the formation of excessive fibrous connective tissue.

Los efectos antiinflamatorios del AH se han demostrado en una serie de estudios in vitro e incluyen inhibición de la quimiotaxis de granulocitos, macrófagos y migración de linfocitos, así como la reducción de la fagocitosis por granulocitos y macrófagos (Ghosh P 1993; Balazs EA et al.;1973; Brandt KD,.1974; Forrester JB et al., 1980; Partsch G et al., 1989).

The anti-inflammatory effects of the HA have been proven on a series of in vitro studies, and they range from inhibition of the granulocyte chemotaxis, macrophages and lymphocyte migration, to the reduction of granulocyte phagocytosis and macrophages (Ghosh P, 1993; Balazs EA et al., 1973; Brandt KD, 1974; Forrester JB et al., 1980; Partsch G et al., 1989).

A reduced effect on the interaction of the enzymes, antigens or cytokines with target cells has been proven (Forrester JV et al., 1981; Ogston AG et al., 1961).

The HA inhibited the neutrophil-mediated degradation; and it has been proven efficient in reducing the production of prostaglandin (PG)E2 by the interleukin-1-stimulated rabbit chondrocytes (Tamoto K et al., 1993; Akatsuka M et al., 1993).

On a controlled clinical trial, the HA treatment reduced the PGE2 and cyclic adenosine monophosphate (cAMP) levels of the synovial fluid. These studies suggest that the anti-inflammatory properties of the HA can be attributed, partly, to its capacity to reduce the production of pro-inflammatory soluble mediators (Punzi L et al., 1989.)

En un ensayo clínico controlado, el tratamiento con AH redujo los niveles de PGE2 y de AMP cíclico del fluido sinovial. Estos estudios sugieren que las propiedades

antiinflamatorias de AH pueden atribuirse en parte a su capacidad para reducir la producción de mediadores inflamatorios solubles (Punzi L et al.; 1989).
The stanozolol shows an anti-inflammatory effect, inhibiting the production of pro-inflammatory mediators in the normal and in the IL-1β treated chondrocytes (Tung JT et al., 2002).
It has also been proven in cultured chondrocytes a significantly reduced expression of the catabolic genes (mRNA) MMP-13, MMP-1, IL-6 and COX-2 both with and without presence of an inducing factor (interleukin IL-1β) in comparison with controls (Richardson CW and Dodge GR, 2000).

Se demostró también en condrocitos cultivados, una expresión significativamente reducida de los genes catabólicos (ARNm) MMP-13, MMP-1, IL-6, y COX-2 tanto con o sin presencia de un inductor (interleuquina IL-1β) en comparación con los controles (Richadson CW and Dodge GR; 2000).
A recent study shows that the stanozolol has chondroprotective effects through the downregulation of genes for pro-inflammatory/catabolic cytokines and enzymes associated with OA (Martins MC et al.; 2018).
Stanozolol reduces apoptosis in equine chondrocytes in vitro by reducing the production of nitric oxide and stimulating IGF-1 production (Saleri et al., 2004).
This matches the evidence presented on muscle. The stanozolol provides protections against acute exercise-induced oxidative stress by reducing the reactive oxygen species (ROS) production, in association with a preservation of the mitochondrial

Employing a world-accepted technique for experimental investigation of OA (Oakley et al. 2004), the intra-articular effect of stanozolol has been proven to reduce the osteophytes formation and the reaction of the subchondral bone, as well as promoting the regeneration of the articular cartilage (Spadari et al., 2013).
The bone mineral density and the biomechanical properties are strongly affected by the effect of the glucocorticoid-induced osteoporosis. On a trial where rats are treated with prednisone acetate chronically, a decrease of the bone density of 20% ($P<0.01$ to $P<0.05$) is induced, and the biomechanical properties are affected 17.1% ($P<0.05$). This is evaluated through a densitometry in both femurs and in the 5th lumbar vertebra. The stanozolol treatment reverts this diagnosis, overcoming in more than 70% ($P<0.05$) both the density and the biomechanical parameters (Liao et al., 2003).

In conclusion, the integration of the two components of **VISSAGRAN**® ® fulfills the three premises:

- **Lubricant function increase**
- **Inflammation interruption**
- **Damage recovery**

And, indirectly, causes the analgesic effect required.

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