STUDY OF ANGIOGENESIS AFTER THE EXPERIMENTAL ACUTE CHOLECYSTITIS

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Summary

The experimental study aimed to induce the inflammation of the cholecyst in 40 pigs, injecting L-α-Phosphatidylcholine in the gallbladder using laparoscopy and to establish the presence of angiogenesis in the healing process. Some groups received then Diclofenacum and some groups did not, until the laparoscopic cholecystectomy, which was performed after 3 or 7 days. Hematoxylin and eosin stains were done from the gallbladder sections. At the reintervention moment there were noticed many adhesions. The histology emphasized the gallbladder’s inflammation, collagenolysis and vascular alteration. When the action of L-α-Phosphatidylcholine decreases (or disappears), vascular repair processes are very active and the edema disappeared, making it safe to start the repair processes. In the area where repair processes are well represented, many blood vessels are present, they are neoformation vessels. Histopathological examination proved that at 3 days L-α-Phosphatidylcholine produced a severe inflammation of the gallbladder’s wall and comprised all of his structures from the surface to depth with marked vascular changes and recent thrombus in some small vessels. At 7 days after administration of L-α-Phosphatidylcholine histopathological examinations show in mid-depth repair processes presenting active fibroblasts, thin collagen fibers and neoformation vessels.

Keywords: Cholecystitis, inflammation, angiogenesis, L-α-Phosphatidylcholine, Diclofenacum

Introduction

The repair process of injured tissue consists of several often overlapping phases with distinct phisiopathological characteristics. Wounded tissue reacts initially with bleeding and vasodilatation. After the initial haemostatic response that results in clot formation, acute inflammation takes place with inflammatory cells invading the injured tissue and surrounding stroma. The next step is the formation of granulation tissue, which serves as a scaffold for angiogenesis and reepithelialization, here fibroblasts having an important role (Seed et al., 2008).

The process of tissue repair requires intense proliferation, which in turn places demands on the nutrition and oxygen supply in the tissue surrounding the injury. This shortage is corrected by angiogenesis, resulting in neovascularization of the inflammatory microenvironment and granulation tissue. Angiogenesis is promoted by vascular-endothelial growth factor (VEGF) and its main receptor on endothelial cells is VEGFR2. Angiogenesis is recognized as the growth and remodeling process by which an initial vascular system is modified to form a complex branching network, characteristic of matured vasculature (Grunewald et al., 2006).

Evidence has been gathered regarding the association between angiogenesis and inflammation in pathological situations. These two phenomena have long been coupled together in many chronic inflammatory...
disorders with distinct etiopathogenic origin, including psoriasis, rheumatoid arthritis, Crohn’s disease, diabetes, and cancer. Lately, this concept has further been substantiated by the finding that several previously established noninflammatory disorders, such as osteoarthritis and obesity, display both inflammation and angiogenesis in an exacerbated manner. (Enzerinka et al., 2010)

The experimental study aimed to induce the inflammation of the cholecyst in pigs, injecting L-α-Phosphatidylcholine in the gallbladder using laparoscopy and to establish the presence of angiogenesis in the healing process after the acute cholecystitis (Stancu, 2011).

Material and methods
The experimental research project included four groups of 10 female pigs (Suis scrofa), with 30-35 kg weight, maintained on standard laboratory conditions.

In the first operative time was accomplished the induction of the acute cholecystitis, by the laparoscopically injection into the gallbladder’s fundus of the L-α-Phosphatidylcholine (Laph) after the initial application of clips on the cystic duct. In all groups on 5 pigs the laparoscopic cholecystectomy (CL) was performed after 3 days and in the other 5 pigs after 7 days.

In the first group of pigs (Lot 1) 2 bottles of 250 mg L-α-Phosphatidylcholine (Laph) were injected on every experience animal’s gallbladder, achieving in this way a concentration of 1 mmol/l.

In the second group of pigs (Lot 2) 1 bottle of 250 mg Laph was injected on every experience animal’s gallbladder, achieving in this way a concentration of 0.5 mmol/l.

In the third group of pigs (Lot 3) 2 bottles of 250 mg Laph were injected on every experience animal’s gallbladder, achieving in this way a concentration of 1 mmol/l. Then, until the CL, one ampoule (3 ml) of Voltaren® (Diclofenacum sodium) of 75 mg was injected intramuscular at 12 hours.

In the fourth group of pigs (Lot 4) 1 bottle of 250 mg Laph was injected on every experience animal’s gallbladder, achieving in this way a concentration of 0.5 mmol/l. Then, until the CL, one ampoule (3 ml) of Voltaren® (Diclofenacum sodium) of 75 mg was injected intramuscular at 12 hours.

During the second intervention, CL, the gallbladder was removed and sections were sent for histopathological examination. Hematoxylin eosin staining were done from sections of the gallbladders and then their aspects were studied with the microscope (Stancu et al., 2011).

Results
In the first group, after 3 days (Sublot 1a) the lesions were pronounced and encompass all the layers.

There are processes of colagenolysis and vascular alterations, more pronounced in small vessels (thin walls) of chorion mucosa. Edema of the chorion is very noticeable throughout the wall thickness, but especially in the external part where it is accompanied by a brutal colagenolysis.

In the upper half part the colagenolysis processes here are also relatively advanced, but they don’t have the magnitude of those from the external half part. Processes of colagenolysis doesn’t include only thin collagen fibers in the chorion but also thick ones from the depth which appear fragmented, turgid, with damage of the tinctoriality.

Lesions of different intensities are found in most blood vessels, regardless of size or type (arteries or veins). Some of them, despite having advanced lesions, can still be estimated, the structural components, especially in larger arteries, although in their case the lumen is almost entirely occupied by debris that tend to organize in an embolus which blocks practically the lumen, incapacitating the vessel. There are arteries in the later stage of alteration, in which none of the
components cannot be assessed but partly their shape and debris resulting from the degradation of embolus tend to mix with those resulting from the degradation of the vessel’s components, with a final tendency of homogenization.

Veins have almost all the lumen blocked, either with the fibrin networks which include a small number of red blood cells or with a material derived from proteins degradation to which no one can appreciate the structural elements.

In the first group at 7 days (Sublot 1b) lesions are more moderate, especially in the deep half part. There are many fibroblasts showing that the reparative processes are in progress. There are many vessels, mostly of small caliber which by their appearance and distribution seem to be neoformation vessels.

Some vessels appear slightly congested and we can observe the initiation of some discrete repair processes. Collagen synthesis processes are in an early stage, fibroblasts are present in small number and performing a certain synthesis activity.

In the second group at 3 days (Sublot 2a) we found relatively pronounced lesions, but not as brutal as in animals that were given two bottles of Laph.

There are processes of colagenolysis on the full thickness, but they are much more pronounced in the mid-depth where practically only from place to place can be noticed fragments of fibers which keep insomuch a specific tinctorial affinity of collagen.

Surface blood vessels present deterioration of the wall and many of them have a stucked lumen with a homogeneous material, where it cannot be distinguished quantifiable morphological structures.

The vessels that still retain a morphological aspect are in the deepest area on a narrow area, but they also have a pronounced congestion, with the lumen filled with erythrocytes.

In the second group after 7 days (Sublot 2b) after the administration, the effect of 1 bottle Laph seems to have disappeared completely and repair processes are present which tend to restore the affected areas.

There are noticed changes from the surface to depth, with the maintaining of the epithelium disposal on relatively large areas but there are some areas with epithelial denudation on the surface. In mid-deep lesions are less obvious, observing the initiation of repair processes that include very large areas. There are many fibroblasts showing that repair processes are underway.

In the area where repair processes are well represented, many blood vessels are present, they are neoformation vessels.

In the third group after 3 days (Sublot 3a) the lesions are very pronounced.

The lesions are largely comparable to those of first group except that the mucosa still presents epithelium on lining relatively large areas although it is modified.
Fig. 2 Neoformation vessels, evidence of the repair processes – Sublot 1b (Goldner’s trichrome stain, ob. 40X)

Fig. 3 Degenerative lesions of the vessels from the surface – Sublot 2a (Goldner’s trichrome stain, ob. 10X)

Fig. 4 Repair processes – epithelial ribbon, numerous fibroblasts and neoformation vessels – Sublot 2b (Goldner’s trichrome stain, ob. 16X)

Alterative processes of blood vessels are present but are not as advanced as on the first group, in the upper half of mucosa. Vessel wall presents modifications so pronounced that they seem irreversible in both large caliber vessels and small vessels. Blood vessels, those with thicker walls, arterioles, presents advanced changes but we still can size up some of the components of the wall, even if by their appearance seem to evolve and not to stop these alterative processes. For vessels with thinner walls, venules, the changes are advanced but here also can be seen as entities some epithelial cells, the outline of the vessel, and in some of them even some cells.

Collagen fibers appear comprised by collagenolysis but some of them can be assessed on relatively large distances especially the thicker ones. Components of muscle, smooth muscle cells can also be much appreciated as entities even though they appear all covered by degenerative and alterative processes.

The edema is also pronounced here, and interfascicular but especially near the
vessels is present a moderately cellular infiltration with mononuclear cells.

In the third group after 7 days (Sublot 3b) there are still present changes in the entire wall thickness. On the mucosa lesions are pronounced, denudation is present on the most of the mucosa. Only on small areas can be seen portions of epithelium on relatively small surfaces. In-depth repair processes are present instead. The statement is supported by the presence of a large number of fibroblasts and thin collagen fibers, numerous vessels, mostly small.

![Fig. 5](image5.png)

**Fig. 5** Injury of the vessels in the upper half part of the cholecystic wall - Sublot 3a (Goldner’s trichrome stain, ob. 10 X)

Large vessels, affected in the first instance, tend not to repair. Reparatory processes are present in most of the wall thickness occupying 60-70% and obviously tending towards, advancing to the upper area of the wall.

Issues identified at 7 days show that the area has been revascularized, the first step in recovery, neoformation vessels entering the area and then the fibroblasts started collagen synthesis. It seems now the only way to repair the conjunctiva. No issues were found regarding the epithelial repair.

It was noted that lesions in the mid-depth evolves more brutal but we noted that even when they trigger reparatory processes, they seem to begin from the most profound area and step forward toward the surface step by step.

![Fig. 6](image6.png)

**Fig. 6** Aspect of the profound part of the cholecystic wall with numerous fibroblasts, collagen fibres and neoformation vessels - Sublot 3b (Goldner’s trichrome stain, ob. 40X)

In addition to endothelial cells involved in angiogenesis processes in this area are more fibroblasts and collagen synthesis processes are in a slightly more advanced stage than in the following areas.

In the fourth group is found after three days (Sublot 4a) we found alterations which comprised all layers, all parts, but in a less advanced stage as the third group. Epithelium is spoiled on the entire surface but it's present.

On small areas epithelial injuries and edema are more discreet, although pronounced, is somewhat tempered. Edema is pronounced, colagenolysis present but not very advanced.

The alterative processes seem to progress. Diclofenacum cannot stop the action but delays Laph effects depending on dose.
There are also vascular alterations but here mainly processes of congestion and not brutal edema and major alterations. In some areas congestion is worse but yet is not accompanied by extravasations of red blood cells. In these areas are present fibroblasts and a moderate number of thin collagen fibrils which shows that reparative processes are present, and which runs for this time at a medium level.

In the fourth group after 7 days (Sublot 4b) the lesions are still present in all components, but repair processes are accompanied by different intensities from one area to another.

The reparatory processes are comparable with those from the 3rd Lot but are in an advanced stage, here being thicker collagen fibers and more discrete near the surface. In addition discrete processes of epithelial repair can be observed on areas not too large, the majority occupied by cells less high than those normally found in the gallbladder.

The presence and appearance of cells suggests the development of epithelial repair processes that are still in early stages and does not occupy large areas. They appear to have an evolutionary character so that in those cases repairing of the epithelium is possible but we cannot appreciate the percentage of the surface where epithelium will be formed (Stancu, 2011).

**Discussion**

Histopathological findings from Sublot 1a, which were given two bottles of Laph presented changes after 3 days on the VB’s wall of a special brutality and include all its components from depth to the surface.

Vascular disorders are particularly pronounced. The consequence is choking all of structures with edema fluid which comprises the other components and prevents their nutrition and good functionality.

Many vessels have altered walls, the circulation being in a very large proportion compromised, the other changes appearing accordingly. Some small vessels from the lamina propria present recent thrombus in
the lumen. Myolysis processes are present in the muscular layer which shows the progression of lesions to the mid-depth, being able to correlate the severity of inflammation produced by sublot Laph.

Mucosal folds present epithelial necrosis and mucosal folds on mural, shows the presence of numerous neutrophils signs of inflammation.

Subserous layer presents connective tissue with blood vessels whose lumen is completely blocked by clots or debris. Practically there are severe changes in all layers of gallbladder’s wall due to chemical aggression of Laph brutal inflammatory phenomena with the consequences of the above.

It seems that the action of Laph is exercised principally on blood vessels and other components change is a consequence of vascular disorders. This statement is supported by the fact that after the administration Laph most drastic changes occurred in the deep area of the wall where the vessels are slightly larger size, where they penetrate in the thick wall, but also the repair process starts here.

This means that when the action of Laph decreases (or disappears), vascular repair processes are very active and the edema disappeared, making it safe to start repair processes.

Histopathological findings from Sublot 3a shows that the administration of 150 mg of Diclofenac succeeded to temper the inflammatory action of Laph in a very limited extent Laph, at 3 days after injection.

The only difference that suggests the protective action of Diclofenac is found in blood vessels in the upper area which seem to be less affected than in the group not taking Diclofenac.

Mucosal epithelium is present in quite large areas, but are present also areas of epithelial necrosis with mucosal denudation. The chrorion is congested with loose connective tissue edematiate, most thrombosed vessels but some still permeable. Muscle layer is narrow with aspect of myolysis, especially of connective and elastic fibers. Subserous layer is heavily edematiated with altered structural vessels.

Histopathological aspects of the subgroup 3b shows that after the 7 days the differences are much more visible, meaning that repair processes here include larger areas (60 -70% of the surface) and have the same tendency to extend to the surface.

Moreover, on the largest area these repair processes are in a more advanced stage, compared with the without Diclofenac administration, there are even parts where appear thicker collagen fibers reinforced. Although their number is not very large, it appears clear the trend of conjunctiva consolidation with progression to fibrosis.

Considering compared the two situations we can conclude that Diclofenac moderates somehow Laph action reducing the inflammatory infiltrate, but is unable to cancel it. Its administration proved to be beneficial, aspect that came out more after seven days.

Histopathological aspects of the fourth subgroup 4a, shows that at 3 days after taking a bottle of Laph and 150 mg Diclofenac daily; the epithelium is totally affected but with a smaller denudation compared to the Sublot 3a. Inflammatory process is present but with a moderate intensity due to the action of Diclofenac.

In chrorion the important edema is noted and colagenolysis, moderate injuries. Subserous vessels are congested tart dishes are congested. A small part of them are thrombosed. There are fibroblasts and collagen fibers sign of the initiation of repair processes.

Histopathological aspects of subgroup 4b shows that at 7 days, there are ongoing repair processes of various intensities, more advanced than in subgroup 3b. On the surface it appears on some areas, a thin epithelial layer and in depth in the subserous layer appears collagen fibers, and blood vessels. Tunica muscle layer is of
fibro-muscular aspect with predominant connective fibers (Stancu et al., 2011).

Inflammation is crucial to defend against pathogens, like in our case Laph. However, longstanding inflammation results in adverse effects. These, in turn, are further enhanced by the activation of angiogenesis. Several evidences demonstrate that chronic inflammation and angiogenesis can be upheld by the same stimuli. Accordingly, several growth factors and cytokines have been associated with both these processes within the literature, further emphasizing the close partnership that gathers them together. Strikingly, these two processes seem to depend on each other.

Common molecular mechanisms have also been found, corroborating this assumption. It is quite remarkable to realize that a wide range of disorders presenting much distinct etiopathogenic origin carry identical molecular mechanisms that cluster inflammation and angiogenesis together (Szekanecz et al., 2007).

This emergent knowledge led to the chase of direct therapeutic approaches against both angiogenesis and chronic inflammation. Targeting new blood vessel formation holds the promise of decreasing influx of immune cells, reducing production of inflammatory and proteolysis mediators, and preventing nutrients supply to an active inflammatory process. Conversely, targeting inflammation would also negatively affect blood vessel formation as described above. However, the intricate interplay between these two processes, discussed in the present paper, blurs the search for therapeutic strategies. Therefore, a careful understanding of the cross-talks between chronic inflammation and angiogenesis must be highlighted for any pathology, aiming at more effective therapies (Eming et al., 2007).

Nonetheless, as epidemiological and experimental evidence indicates that dietary compounds, such as polyphenols, offer protective effects against both inflammation and angiogenesis, the relevance of natural agents as chemo preventive against disorders associated with chronic inflammation and angiogenesis should also be taken into account, especially because they are easier and cheaper to establish. However, there is still one question remaining to be answered regarding the interplay between chronic inflammation and angiogenesis: are they cause or consequence (Costa et al., 2007)?

Fibroblasts from patients with rheumatoid arthritis (RA) or osteoarthritis (OA), as well as fibroblasts from healthy synovial tissue and healthy skin, were cultured and subcutaneously engrafted into immunodeficient mice. Cell infiltration and angiogenesis were analyzed in the grafts by immunohistochemical studies.

Inflammatory (OA and RA) synovial fibroblasts, compared with healthy dermal or synovial tissue fibroblasts, induced a significant enhancement in myeloid cell infiltration and angiogenesis in immunodeficient mice. These results demonstrated that chronic synovial inflammation is associated with stable fibroblast changes that, under hypoxic conditions, are sufficient to induce inflammatory cell recruitment and angiogenesis, both of which are processes relevant to the perpetuation of chronic inflammation (Muller-Ladner et al., 2007).

An important contribution of the hyperplasia of fibroblasts to chronic inflammation and tissue destruction has also been proposed, particularly in patients with rheumatoid arthritis (RA), in whom there is sufficient evidence to indicate an association between abnormal fibroblast phenotype and chronic inflammation.

The contribution of a pathologic fibroblast phenotype to the development of tumors is widely recognized. Tumor stroma has the capacity to induce the recruitment of bone marrow–derived myeloid cells that, in concert with cancer fibroblasts, induce a strong angiogenic response fostering tumor growth (Buckley et al., 2007).

Therefore, chronic inflammatory and tumor environments could similarly
favor myeloid cell recruitment and angiogenesis through phenotypic changes in stromal fibroblasts.

Increasing evidence points to a central link between inflammation and activation of the stroma, especially of fibroblasts therein. However, the mechanisms leading to such activation mostly remain undescribed (Hinz et al., 2007).

Conclusions

Histopathological examination proved that at 3 days L-α-Phosphatidylcholine produced a severe inflammation of the gallbladder’s wall and comprised all of his structures from the surface to depth with marked vascular changes and recent thrombus in some small vessels.

At 7 days after administration of Laph, histopathological examinations show in mid-depth repair processes presenting active fibroblasts, thin collagen fibers and neoformation vessels.

After 3 days after the administration of Laph, during the acute cholecystitis there are no signs of repair processes and angiogenesis. Only at 7 days, the chronic inflammation triggers repair processes and the beginning of angiogenesis.

Although many disorders of distinct etiopathogenic origin have identical molecular mechanisms that cluster inflammation and angiogenesis together, in our study the angiogenesis is the result of the repair processes after the experimental induction of inflammation.

References


