THE INFLUENCE OF DICLOFENACUM ON THE EXPERIMENTAL ACUTE CHOLECYSTITS

B. Stancu¹*, V. Micăuş², O. Andercou¹, A. Osiceanu³

¹ SECOND SURGICAL DEPARTMENT, UNIVERSITY OF MEDICINE AND PHARMACY “IULIU HAŢIEGANU”, CLUJ NAPOCA; ² HISTOLOGY DEPARTMENT, FACULTY OF VETERINARY MEDICINE, UNIVERSITY OF AGRICULTURAL SCIENCES AND VETERINARY MEDICINE CLUJ NAPOCA; ³ ANATOMY DEPARTMENT, FACULTY OF MEDICINE AND PHARMACY, ORADEA UNIVERSITY

Summary

The experimental study aimed to induce the acute alithiasic cholecystitis in 40 pigs, injecting L-α-Phosphatidylcholine in the gallbladder using laparoscopy and to establish the role of Diclofenacum on the acute cholecystitis, after intramuscular administration.

After the induction some groups received Diclofenacum until the laparoscopic cholecystectomy, which was performed after 3 or 7 days. Hematoxylin and eosin stain were done from the gallbladder sections. At the reintervention moment there were noticed many gallbladder-epiploic, gallbladder-gastric, gallbladder-enteral and parietal-gallbladder adherences. Macroscopic, the intensity of the gallbladder and near gallbladder inflammatory process was strongly marked in the group which was operated later making more difficult the cholecystectomy. The histology emphasized the gallbladder’s inflammation, colagenolysis and vascular alteration. The experimental induction of the acute acalculous cholecystitis is possible by injecting using laparoscopy into the gallbladder L-α-Phosphatidylcholine in concentration over 0.5 mmol/l, the marked inflammatory lesions being emphasized also macroscopic and microscopic, but much decreased in animals who received Diclofenacum.

Key words: acute cholecystitis, L-α-Phosphatidylcholine, laparoscopic cholecystectomy, inflammation, Diclofenacum

bstancu7@yahoo.com

Introduction

The acute cholecystitis (AC) is one of the frequent complications of gallbladder’s (VB) lithiasis. In 10% cases we also found the acute acalculous cholecystitis (AAC). The AC presents higher technical difficulties than chronical cholecystitis because of the intensity of the inflammatory process and of the pericholecystic adherences which lead often to conversion.

Many authors recommend as the most favourable operative moment for laparoscopic cholecystectomy (CL) for the AC, the first 48 – 72 hours from the beginning. (Teixeira et al., 2002 )

In this study we used L-α-Phosphatidylcholine (Laph) also known as L-α-Lecithin for the experimental induction of the AC.

This substance, a hydrolysis product of Lecithin, was chosen because it is a normal constituent of bile and because of the remark of it’s concentration increase in the VB of patients with AC. Laph has cytolythic features and of membrane perturbation, being capable to induce the acute inflammation of the VB on experience animals (rabbits, guinea pigs, cats,etc.). (Neiderhiser, 1986)
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. The operatory disposal was with the surgeon being placed on the left side. Beside him, at the distal extremity of the animal was the cameraman, and on the opposite side the assistant surgeon. For laparoscopic approach were used 4 surgical trocars: 2 trocars of 10 mm and 2 trocars of 5 mm. In the first operative time was accomplished the induction of the AC, by the laparoscopically injection into the VB at the fundus level of the Laph after the initial application of clips on the cystic duct. In all groups on 5 pigs the 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 Laph were injected on every experience animal’s VB, 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 VB, 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 VB, 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 VB, 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 VB was removed and sections were sent for histopathological examination. Hematoxylin eosin staining were done from sections of the VBs and then their aspects were studied with the microscope. (Stancu, 2008, 2009, 2011a)

Results

In the first group, after 3 days (Sublot 1a) the lesions were pronounced and encompass all the layers. On very large areas the epithelium is missing, with the modification of the normal aspect of the folds in shape and also in size.

On some areas the epithelium was still present but presented changes whose intensity was relatively high, the lesions are irreversible, until the breakup of the epithelium and the denudation of those areas is just a question of time. There weren’t highlighted areas with normal epithelium.

Wrinkles form retains a certain degree but some are vestigial, and the components of the structure are all comprised by degenerative and pronounced alterative processes at different stages.

There are also 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 tintoriality.

There are also processes of myolysis that seem to have progressive nature and tendency to generalize. Muscle cells are also included on advanced alterative processes that seem irreversible, although regarding their appearance they seem the most resistant of all components (declines last).

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 can not 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. (Fig. No. 1)

Veins have almost all the lumen blocked, either with the fibrin networks which include a small number of red blood cells (RBCs) 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. In the mid superficial part the changes are still advanced, the epithelium still appears affected and presents the tendency to detach from the basal membrane so that large areas looks denudated. In mid deep part the situation is totally different, here the lesions largely ceded and we can notice the initiation of reparative processes which even if they aren’t carried out at the same intensity, they cover very large areas.

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. (Seed, Walsh, 2008)

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 ceratin synthesis activity.

There are differences from one area to another. In the area where repair processes are underway, they comprise 25% to 60% of surface depending on the examined area.

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.

Changes were identified from the surface to depth, with the maintaining of the epithelium as disposal on relatively large areas, but although the cells are not yet detached and are still closely side-by-side, they are totally damaged, and is only a matter of time until they fall off.

Actually, there are also areas where the epithelium tends to fall, to form flaps, large groups of cells; edema is pronounced both in the upper half but especially in the deepth. 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 can not be distinguished quantifiable morphological structures. Muscle cells are covered by myolysis and only in some of them we can assess the form, less structural details, tending to structural uniformity inside. 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. Repair processes comprise 35% to 70% of the surface, depending on the location of the area examined.

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.

In mid profound the alterations are well advanced, the lesions are irreversible, they are generalized but in a less advanced stage as on the first group. All the structures are compromised in the half deep, so that we can hardly assess their structure.

From the collagen fibers remained only small fragments but these are also rare and on the blood vessels we can assess only the contour, all the structures being spoiled and the lumen occupied by networks that appear to be fibrin.

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.

In the upper half alterative processes are present but are not as advanced as in the half deep, in some areas the epithelium is present, but there is so affected that by the total denudation is just a matter of time.

Collagen fibers appear comprised by colagenolysis 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 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.

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. No. 2)

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, collagenolysis present but not very advanced.

The alterative processes seem to progress. Diclofenac can not stop the action but delays Laph effects depending on dose.

The difference compared to cases with only 2 doses of Laph is that approximately 15-20% of wall thickness, the deepest part, to the liver bed, present discrete alterations largely keeping its components.

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 extravasation 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. (Fig. No. 3)

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 on approximately 70% thickness 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 VB.

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 can not appreciate the percentage of the surface where epithelium will be formed. (Fig. No. 4) (Stancu, 2011)

**Disscusions**

Tissue inflammation is a direct response of tissue to external stimuli, as is Laph in our experimental study, and serves to locate and isolate the injured tissue area, protecting the surrounding healthy tissues.

![Fig. 1 Impaired small-caliber vessels in the general protein degradation process - Sublot 1a](image1)

![Fig. 2 Extensive denudation of the mucosa, pronounced edema - Sublot 3b](image2)
It tends to neutralize and inactivate toxic substances by humoral factors and enzymes. Also destroy and the limited the growth of infectious microorganisms and counteract their effect, preparing the area for healing and repairing by removing devitalized tissue and debridated cells.

In the early days of the development of AC, VB’s wall is moderately or significantly thinned, the main feature being the hemorrhagic edema that is most obvious in the subserosa. There appears ulcerations of the mucosa and small areas of necrosis in the wall depth.

At the peritoneal surface fibrinous exudate is formed resulting then a matrix of fibrin, so that fibrinous adhesions can be found between VB and adjacent viscera. This phenomenon of formation of adhesions occurs when two surfaces of the injured peritoneum are in apposition.

Surgical or inflammatory tissue lesions reduce or eliminate the blood flow, thereby producing ischemia leading to local persistence of the fibrin matrix by suppressing fibrinolysis. This matrix is gradually replaced by vascular granulation tissue containing macrophages, fibroblasts and giant cells. The adhesions are matured in fibrous bands containing usually lumps of calcification. Adhesions are often covered by mesothelium containing blood vessels and connective tissue fibers, including elastin. (Dix et al., 2003)

From the microscopic point of view, in the initial stage of the AC, the most important change is the acute inflammatory reaction where predominant are hemorrhage and edema. Leukocytes, although present in moderate numbers, are not as numerous as would be expected in a vascular reaction so severe.

Various experiments have shown that a similar type of acute inflammatory reaction in which the granular leukocytes are dispersed, can be produced by injection of bile directly into the tissues. This comes to the idea that AC is primarily due to the action of bile on VB’s wall.

In addition to areas of necrosis are occasionally encountered small intramural abscess. The exudate is rich in fibrin, but after 2-3 days of development is rapidly infiltrated by fibroblasts and capillary balls. Thrombi can also be found occluding few
arterioles and ischemia may be responsible for much of necrosis.

The inflammatory edema and distension of the VB can interfere with blood flow to the wall so that will result in small areas of infarction, especially in the fundus where blood flow is the weakest.

If there is a very extensive necrosis of the wall, when we talk about producing gangrenous cholecystitis, characterized by total destruction of mucosal and muscular layer, with a significant risk of perforation and biliary peritonitis.

When the content of VB is cloudy or appears to be frankly purulent the empyema of VB is present. In many of these cases, the content is actually not foul, but thickened bile by inflammatory exudate mixture and thus turned cloudy in appearance, mainly due to precipitation of calcium carbonate and cholestrol. Occasionally, VB empyema appears as the AC suppurative sequel.

Early in the first week of AC, there is a considerable infiltration with neutrophils of VB’s wall. Small intramural abscesses can be formed while still organizing inflammatory exudate and serous adhesions. In those cases in which tissue damage was not severe the inflammatory process begins to diminish.

During the third week have been gradually replaced neutrophils by lymphocytes, plasma cells, eosinophils and macrophages. Abscesses and necrotic areas of the wall, some of which contain dark brown or black biliary material, become surrounded by granulation tissue containing many macrophages with foamy cytoplasm in which are present brown pigment grains.

Granular tissue organization is still active in this period leading to a more or less extensive fibrosis of the VB’s wall and replacing all or part of the muscular layer with this fibrous tissue. Ulcers will heal with subsequent appearance of a simple flat epithelial layer of regeneration.

Focal necrosis with xantoma-like collections of foamy macrophages may last long time in the fibrous tissue poor in cells. Collections of lymphocytes may also persist in some cases forming lymphoid follicles, usually containing germinal centers, this condition being described as follicular cholecystitis.

The mucous membrane is denudated on the variable surfaces in the acute phase, is regenerated to cover the granulation tissue, but its normal appearance with intersecting folds is lost forever. (Rosai, 2004)

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 VB’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 chorion is congested with loose connective tissue edematitate, 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 edematitate with altered structural vessels.

Histopathological aspects of the Sublot 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 Sublot 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 chorion the important edema is noted, colagenolysis and moderate injuries. Subserous vessels 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 Sublot 4b shows that at 7 days, there are ongoing repair processes of various intensities, more advanced than in Sublot 3b. On the surface it appears on some areas, a thin epithelial layer and in depth the subserous layer appears collagen fibers, and blood vessels. Tunica muscle layer is of fibromuscular aspect with predominant connective fibers. (Stancu, 2011 b)

Conclusions

The experimental induction of the AAC is possible by injecting using laparoscopy Laph into the VB in concentration over 0.5 mmol/l, the marked inflammatory lesions being emphasized also macroscopic and microscopic, but much decreased in animals who received Diclofenacum.

Histopathological examination proved that at 3 days Laph produced a severe inflammation of the VB’s wall and comprised all of his structures from the surface to depth with marked vascular changes, important edema, epithelial necrosis, intramural absceses and neutrophils infiltrate, recent thromus in some small vessels and myolysis.
At 7 days after administration of Laph, histopathological examinations show the substantial inflammatory damage of VB is top half of the wall, but in mid-depth repair processes are present deep to detect active fibroblasts, thin collagen fibers, neoformation vessels, mucosal area covered by a band fibromuscular cord epithelial and appearance of the muscular layer.

Histopathology in experimental animals that received Diclofenac in addition to Laph show that it manages to only slightly reduce the inflammatory action of Laph in any dose, at 3 days after injection, the vessels in the upper part of the VB’s wall being less affected and the epithelium which is not totally destroyed and the repair processes that occur after seven days are in an advanced stage, with thick collagen fibers in some areas with progression to fibrosis.

References
Dix, F.P.; Bruce, I.A.; Krypcyzk, A.; Ravi, S.: A selective approach to histopathology of the gallbladder is justifiable. Surgeon (Edinburgh), 1, 4, 233 – 235, 2003
Stancu, B.; Miclăuș, V.; Andercou, A.; Mironiuc, A.: Histopathological aspects of the acute acalculous cholecystitis experimentally induced by laparoscopy with L-α-phosphatidylcholine. Studia Universitatis Vasile Goldiș (Arad), 19, 1, 53 – 58, 2009