Effects of aspirin on mesenteric lymph nodes of rabbits as basis for its use on lymph nodes metastases[1]

Efeitos da aspirina em linfonodos mesentéricos de coelhos como base para o uso em metástases linfonodais

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ABSTRACT

PURPOSE: To evaluate the effects of aspirin 10% and 20% on mesenteric lymph nodes of rabbits as basis for its use on lymph nodes metastases.

METHODS: A total of 20 lymph nodes from 20 rabbits (randomized in four groups) were evaluated. Aspirin solutions 10% (groups A and C) and 20% (groups B and D) were injected into mesenteric lymph nodes of healthy rabbits and had its gross and histological effects evaluated at 24 hours (groups A and B) and at seven days (groups C and D).

RESULTS: In the groups A and B evaluated at 24 hours it was observed extensive necrosis and hemorrhage, a significant increase in apoptosis throughout the lymph node with medullary sinuses enlargement and an increase in germinal centers. In the groups C and D evaluated at seven days of solution injection there was also an increase in apoptosis with higher elevation of histiocytes and a significant decrease of necrosis and an increase of giant cells was noticed causing a foreign body chronic inflammation. In all comparisons, there were no differences between the concentrations used (10 and 20%).

CONCLUSIONS: The injection of aspirin on lymph nodes caused necrosis and an increase of apoptosis after 24 hours and after seven days of treatment there was regeneration of the lymph nodes, with intense decrease of necrosis and a great elevation of apoptosis. These experimental results support future clinical studies on application of aspirin in the treatment of lymphatic metastases, since the increase of apoptosis is one of the pillars of cancer therapy.

Key words: Aspirin. Lymph Nodes. Neoplasm Metastasis. Apoptosis. Therapeutics. Rabbits.

RESUMO

OBJETIVO: Avaliar os efeitos do ácido acetilsalicílico a 10% e 20% em linfonodos mesentéricos de coelhos para posterior embasamento e uso em metástases linfonodais.

MÉTODOS: Um total de 20 linfonodos de 20 coelhos (divididos aleatoriamente em quatro grupos) foi avaliado. As soluções de aspirina a 10% (grupos A e C) e 20% (grupos B e D) foram injetadas em linfonodos mesentéricos de coelhos sadios e seus efeitos macroscópicos e histológicos foram avaliados em 24 horas (grupos A e B) e em sete dias (grupos C e D).

RESULTADOS: Nos grupos avaliados em 24 horas (A e B) foi verificada intensa necrose e hemorragia, aumento importante de apoptose em todo o linfonodo, com alargamento dos seios medulares e aumento dos centros germinativos. Nos grupos avaliados em sete dias (C e D) também houve aumento da apoptose, com maior elevação de histiócitos e diminuição importante da necrose; a hemorragia foi ausente e aumento de células gigantes foi visualizado, conferindo processo inflamatório crônico do tipo corpo estranho. Não houve diferença entre as concentrações utilizadas (10 e 20%) em nenhuma das comparações.

CONCLUSÕES: A injeção de aspirina em linfonodos causou necrose e um aumento de apoptose após 24 horas e após sete dias de tratamento, houve regeneração dos gânglios linfáticos, com diminuição intensa de necrose e grande aumento de apoptose. Uma vez que o aumento de apoptose é um dos pilares dos tratamentos antineoplásicos, estes resultados experimentais embasam eventual aplicação clínica da aspirina no tratamento de metástases linfonodais,

Descritores: Aspirina. Linfonodos. Metástase Neoplásica. Apoptose. Terapêutica. Coelhos.

Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries[1]. Once a large number of patients have distant metastases on lymph nodes adjacent to the tumor at initial diagnosis and the lymph node spread is a major determinant for the staging and the prognosis of most human malignancies[2], their treatment is needed.

Resection surgery is considered the main type of treatment for cure of lymphatic metastases but in some situations it presents limiting factors such as number and location of lymph nodes involved and especially the adherence to adjacent organs and structures[3,4].

Considering such difficulties and therapeutic characteristics of lymphatic metastases[3,4], new treatment options need to be developed[5]. In order to have applicability it's expected that these new treatment options have effectiveness, low cost, low frequency of side effects and they should be easily executed.

In our research group, we had the opportunity to study and scientifically prove cytolytic effects of aspirin (acetylsalicylic acid) and its derivatives, either *in vitro* or *in vivo* on several experimental models[6-10]. Following this line of research, the purpose of this experimental study was to evaluate the effects of aspirin on normal lymph nodes of rabbits as basis for its later use on lymph nodes affected by tumor.

Methods

The experimental protocol was approved by the Ethics Committee on Animal Experiments of the Institution. The rabbits were kept according to the guidelines of the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 1996) and according to the ethical principles of the Brazilian College on Animal Experimentation (COBEA).

Animal housing, anesthesia, surgical procedures and test solution application

A total of 20 male, rabbits, 14-18 weeks old and weighting 3400-4000g, were studied. The animals were kept in light-dark cycles (12/12h) with free access to food and water; they were deprived of food for six hours prior to the experiments but received water *ad libitum*. All animals were anesthetized by intravenous injection of 10% ketamine hydrochloride (100mg/kg) and 2% xylazine hydrochloride (10mg/kg) and subjected to median laparotomy via supraumbilical incision for direct visualization of the mesenteric lymph nodes.

Subsequently, all animals were randomly allocated via a computer generated process into four groups of five rabbits each and 0,5ml of the test solutions were injected on mesenteric lymph nodes: groups A and C received intranodal injection of 10% acetylsalicylic acid and groups B and D received intranodal injection of 20% acetylsalicylic acid. In order to obtain the desired concentrations, 1g or 2g of acetylsalicylic acid (Pharma Nostra, Anapolis-GO, Brazil) were dissolved in 10ml of 10% sodium bicarbonate, forming bicarbonate acetylsalicylic acid. The solutions were prepared just before use. Finally, the incision was closed, in layers, with 4-0 nonabsorbent nylon sutures.

Early and late evaluations, and clinical evolution

The early effects (groups A and B) of solutions on mesenteric lymph nodes were evaluated by sacrificing rabbits 24h after the end of treatment. The late effects (groups C and D) of solutions on mesenteric lymph nodes were evaluated by sacrificing rabbits seven days after the end of treatment. All rabbits were killed by a lethal intravenous anesthetic dose.

During the study period (variable according to the group), were assessed the clinical outcomes of all animals through objective parameters, including recovery from surgery, food, and activity, being then classified into good, fair, or poor progress.

Gross analysis, collection of specimen and histopathogical examination

After 24 hours (groups A and B) or seven days (Groups C and D) of treatment, all animals were submitted to a second laparotomy in order to dissect the lymph nodes (1 per animal) for histopathogical study. During the laparotomies were carried out gross analysis of all abdominal cavities.

All lymph nodes collected were kept in buffered formol for two days; it was then prepared for mounting on histological slides and colored to hematoxylin eosin. These were analyzed by an experienced pathologist who was blind to the study, in a bright field optic microscope (qualitative analysis). The histological findings were graduated, by pathologist, into cross from the minimum of one cross (+1) to the maximum of four crosses (+4).

Results

Clinical evolution and gross examination of abdominal cavity

Animals present good clinical evolution in all groups without deaths. There was no significant change in weight of the

animals. There were no abnormalities in all abdominal cavities.

Histopathogical evaluation

Table 1 shows the descriptions of the histopathological findings for each group.

TABLE 1 – Histopathological findings distributed according to each group.

Findings	Group A (10% - 24 h)	Group B (20% - 24 h)	Group C (10% - 7 d)	Group D (20% - 7 d)
Necrosis	+++	++	- / ++	- / +
Bleeding	++	+ / ++	Absent	Absent
Histiocytes	+	++	+++	+++ / ++++
Eosinophils	+	+ / ++	+	- / +
Giants cells	Absent	Absent	++	+++
Apoptosis	High (Diffuse)	High (GC and CA)	High (GC and CA)	High (GC and CA)

h = hours; d = days; + = indicates the presence of finding (ranging from + to ++++); - = indicates absence of finding; / = indicates that there were differences between the slides evaluated; GC = Germinal center; CA = Cortical area.

Group A (10%)

Necrosis was intense (+3) as hemorrhage (+2) spread in the lymph nodes (Figure 1). Around the necrosed areas were identified histiocytes and eosinophils (+1), and a significant increase in apoptosis (Figure 2). There was an enlargement of medullary sinuses.

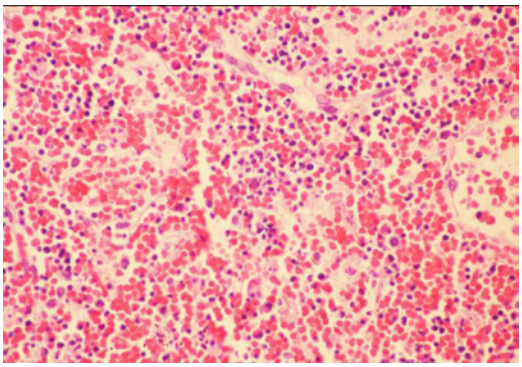

FIGURE 1 – Photomicrograph of surgical specimen from mesenteric lymph nodes revealing intense hemorrhagic necrosis after 24 hours of treatment (group A). (Hematoxylin-eosin, original magnification 100x).

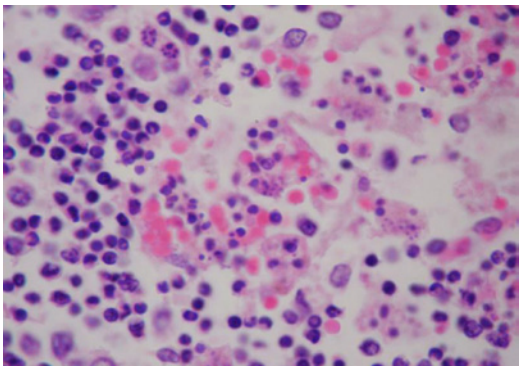

FIGURE 2 – Photomicrograph of surgical specimen from mesenteric lymph nodes showing increased apoptosis around necrotic areas after 24 hours of treatment (group A). (Hematoxylin-eosin, original magnification 150x).

Group B (20%)

Necrosis was identified (+2) in medullar and cortical regions. Hemorrhage varied in intensity from +1 to +2/+4 and histiocytes (+2). The number of germinal centers varied from 18 to 28. As in group A, apoptosis was increased around the necrosed areas (Figure 3).

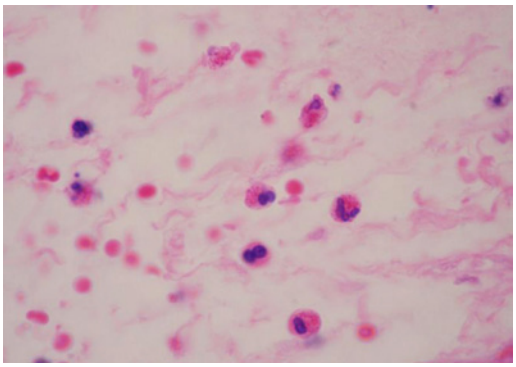

FIGURE 3 – Photomicrograph of surgical specimen from mesenteric lymph nodes showing eosinophils after 24 hours of treatment (group B). (Hematoxylin-eosin, original magnification 150x).

Group C (10%)

Necrosis varied from absent to +2 in the medullary sinuses. Hemorrhage was absent. Around necrosed areas there was an increase in histiocytes (+3). Eosinophils were observed but with just +1 in intensity. There was an enlargement in medullary sinuses (+2) (Figure 4). In 3 cases there were giant cells (+3). Apoptosis increased in medullary sinuses and in germinal centers (Figure 5).

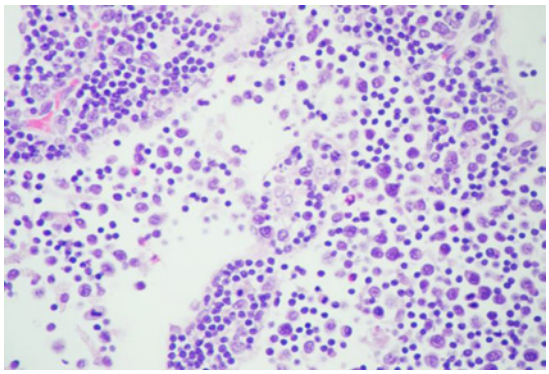

FIGURE 4 – Photomicrograph of surgical specimen from mesenteric lymph nodes demonstrating medullary sinuses enlargement with apoptosis after seven days of treatment (group C). (Hematoxylin-eosin, original magnification 100x).

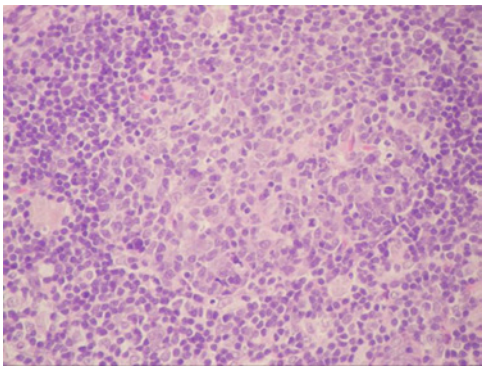

FIGURE 5 – Photomicrograph of surgical specimen from mesenteric lymph nodes revealing germinal center with apoptosis after seven days of treatment (group C). (Hematoxylin-eosin, original magnification 100x).

Group D (20%)

There was a decrease in necrosis (Figure 6), varying since absent until +1 and a decrease in eosinophils (absent to +1). Histiocytes were evident (+3 to +4) (Figure 7). There were 6 germinal centers. Giant cells, as in group C, were observed (+3) (Figure 8). Apoptosis was also increased, especially in medullary sinuses.

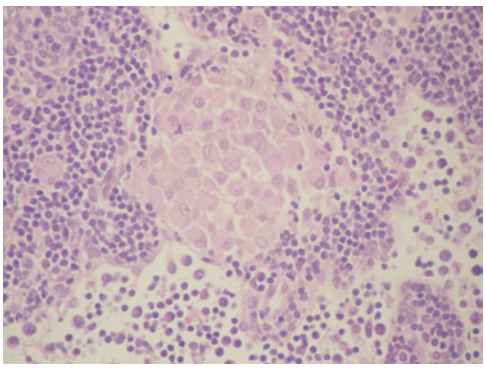

FIGURE 6 – Photomicrograph of surgical specimen from mesenteric lymph nodes showing absence of necrosis and hemorrhage after seven days of treatment (group D). (Hematoxylin-eosin, original magnification 150x).

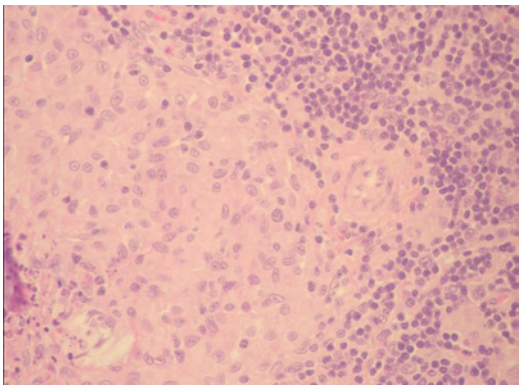

FIGURE 7 – Photomicrograph of surgical specimen from mesenteric lymph nodes showing histiocytes with granuloma formation after seven days of treatment (group D). (Hematoxylin-eosin, original magnification 150x).

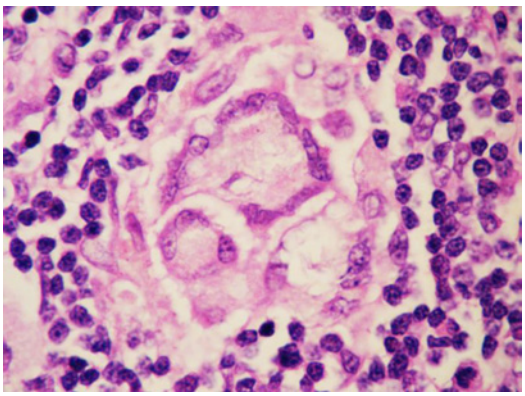

FIGURE 8 – Photomicrograph of surgical specimen from mesenteric lymph nodes showing giant cells after seven days of treatment (group D). (Hematoxylin-eosin, original magnification 150x).

Discussion

Starting with the assumption that lymphatic metastases are determinants of a poor prognosis and their treatment often is not possible[2-4], the search for new therapeutic approaches becomes necessary[5]. Thus, the main focus of this study was to evaluate the effects of aspirin on mesenteric lymph nodes of rabbits, as a basis for therapeutic method for the treatment of on lymphatic metastases.

In this experimental research, there were no differences in the histological results of groups A and B, they were gathered to be discussed. In these groups was evident the necrotizing effects of the aspirin solution and in all lymph nodes there were necrosis. Ohnishi *et al*.[11] found the same lesions using acetic acid on liver and Saad-Hossne *et al*.[7] also found coagulative necrosis on liver of rabbits after the injection of acetic acid and aspirin. In a common pattern of cell death caused by oxygen loss, the necrotic cells show an increase of eosinophils. In the group B the amplification of eosinophils was very important, as a characteristical necrotic

process and acute inflammatory response. This occurs by the increased attachment of eosine to the denaturated citoplasmatic proteins and, also, to basophilic loss usually provided by ribonucleid acid in cytoplasm[12,13]. It was verified the enlargement of medullary sinuses, demonstrating progressive elevation of macrophage activity and inflammatory cell response.

In these groups (A and B), in which the acute response to antigenic stimulus was evaluated, there was an increase of germinal centers, more intense in group B. This is characteristically a follicular hyperplasia, with the germinal centers forming the place of transformation and multiplication of lymphocytes that reacts to antigenic stimulus. The reactive germinal centers are formed by a mixture of cells, like the centroblasts, centrocytes, dendritic cells, small T lymphocytes CD4+ / CD57+ and macrophages, as B lymphocytes in maturation[13-15].

The event considered as of greatest value in this experimental study was the elevation of apoptosis. In groups A and B the elevation of apoptosis occurred in all parts of the lymph nodes. The induction to apoptosis may be caused by DNA damage, whether by direct lesion or by free radicals production, by mitochondrial lesions and also by cellular influx of calcium (ATP dependent) with caspases activation[16,17]. Apoptosis may occur in many situations and has the function to eliminate harmful cells and useless cells[16,17]. It may also be considered a pathological situation when cells suffer injury beyond the repair capacity, mainly when DNA or cells' proteins are affected, time when injured cells are eliminated[16,17].

In groups C and D the effects of aspirin solution were evaluated in 7 days, and were observed the alterations compatible to later inflammatory processes, without difference between both. There was an important decrease of necrosis, with regenerative process, but without fibrosis. As a consequence of apoptosis elevation, the number of histiocytes was also high. The sinusal histiocytes are macrophages found in the spleen and lymph nodes. The macrophages are responsible for phagocytosis[18]. Still compatible to later inflammatory processes, in these groups hemorrhage and eosinophils were absent. Finally, there was an increase in giant cells, with aggregated epithelioid macrophages surrounded by linphocytes, featuring a cronic foreign body inflammation.

The knowledgement of apoptotic pathways has a great applicability in medicine and pharmacology, whether in developing new drugs to induces apoptosis, as a therapeutic strategy, or understanding the mechanisms of radiotherapy or chemotherapy resistance[19-21]. Researches related to apoptosis are really important in practical application of discoveries in diseases related to homeostatic balance loss, such as cancer and neurovegetative diseases[16].

In a recent review[22], the importance of apoptosis in cancer cells was discussed; this can be induced by hypoxia, shortage of nutrients or growth factors, and radiotherapy, or chemotherapy. As a means of protecting the host, physiologic apoptosis is rapidly and specifically recognized by phagocytic cells. Apoptotic bodies are silently removed by phagocytosis; this event is associated with the release of potent antiinflammatory mediators like transforming growth factor-b (TGF-b), prostaglandin E2, or platelet-activating factor to avoid local inflammatory reactions. Apoptosis phenomenon eliminates cells that have accumulated DNA damage without causing inflammation and prevents tumor formation first and tumor growth later.

Once the main result of this study was that in all lymph nodes evaluated were found aspirin-induced apoptosis, and activation of apoptosis is a major goal of cancer therapy[19-21], the authors can assume that aspirin could be beneficial for the treatment of lymphatic metastases.

The regular intake of non-steroidal antiinflammatory drugs, inhibitors of cyclooxygenase (COX), such as aspirin, can reduce the risk of development of some cancers[23-26] and also induce apoptosis in various cancer cell lines[27]. However, the molecular mechanisms through which these drugs induce apoptosis are not well understood[27]; there are several mechanisms proposed, including inhibition of proteasome function, activation of ceramide pathway, activation of caspases, up-regulation of several proapoptotic proteins, down-regulation of NF-κB activity, and generation of endoplasmic reticulum and oxidative stress[27,28].

The overexpression of COX-2 is commonly found in many cancers[29,30]. Several mechanisms by which COX-2 contributes to progression of cancer have been reported, including stimulation of proliferation and inhibition of apoptosis of cancer cells, stimulation of cancer cell invasion and angiogenesis, and suppression of immune responses[29,30]. Targeting COX-2 provides an important opportunity in cancer research; indeed, the expression of COX-2 has been reported as an indicator of poor prognosis in a wide variety of tumors[29,30]. The COX-2 overexpression is causally linked to the suppression of the host's immune system, with an enhanced resistance to apoptosis and stimulation of cancer cell growth and invasion[29,30]. So we may assume that the aspirin use in lymph nodes with tumor cells could stimulate apoptosis and destroy these cells (as observed in this experimental research) and also inhibit the activity of tumor by inhibition of COX 1 and 2. This ability to destroy tumor cells has already been investigated by our group[6-10] in other reports using experimental tumors (Ehrlich

and VX-2) and by others[27,31-38].

Conclusions

Aspirin solution cause necrosis and an increase in apoptosis after 24 hours and that after seven days of treatment there was regeneration of the lymph nodes, with intense decrease of necrosis and a great elevation of apoptosis without difference in the concentration of solution (10 and 20%). Further clinical studies are warranted to evaluate the efficacy of this strategy on lymphatic metastases.

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